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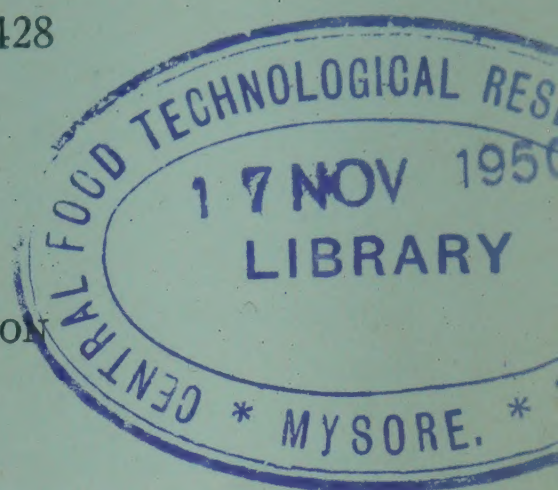
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*This series of papers is the result of an International Conference on Vitamin E, held by the Section of Biology of The New York Academy of Sciences on April 15 and 16, 1949.

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FOREWORD

By Karl E. Mason

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New York*

It gives me immeasurable pleasure to write the foreword to this monograph on Vitamin E. No other vitamin has received such special honor and consideration. No other vitamin has presented such a challenge to students of nutrition. Here we again take our bearings and chart new courses toward the hidden secrets of vitamin E.

It is fitting that due respects be paid to those great pioneers in experimental nutrition whose efforts made possible the advent and cultivation of this mysterious member of the vitamin family—I refer especially to the late F. G. Hopkins, T. B. Osborne, and L. B. Mendel and to E. V. McCollum. Their spirit of uncompromising integrity and generous fellowship in scientific exploration have been a guide to us in the work described here.

Conceived as the X-factor, fostered through several years of gestation *in utero* and *in testiculo* by Mattill and Evans and their associates, and christened VITAMIN E by Sure, in 1924, our subject began a somewhat precarious infancy.

After a more vigorous adolescent growth, its period of puberty was observed in London 10 years ago (April 22, 1939) as a three-session Symposium, organized by Sir Jack Drummond and Mr. Alfred Bacharach, under the auspices of the Nutrition Panel of the Society of Chemical Industry.

That Symposium, and its Proceedings published just as the last war began, did much to crystallize opinion and guide the course of further investigations during the past decade. Continuity between this and the present publication resides in Doctors Charles Engel, Thomas Moore, Evan Shute, and your chairman.

This monograph signifies the early maturity of our subject—actually, its 25th anniversary. We now view a much more complex subject than we saw a decade ago. New facets are to be polished and new spectra are to be revealed. These, we hope, will aid in dispelling the mists that conceal our ultimate goal—a full understanding of the functions and the practical usefulness of vitamin E.

I

MORPHOLOGIC LESIONS IN VITAMIN E DEFICIENCY: INTRODUCTORY REMARKS

By A. M. Pappenheimer

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It is a very great privilege to write the introduction to the opening section of the Vitamin E Monograph. The vitamins, unlike the genes, have, as yet, no political connotations, and we can freely and fruitfully discuss all the varied aspects of the subject.

The program which Dr. Mason has assembled covers many ramifications of Vitamin E research—morphologic, chemical, and therapeutic. The papers in this section have been grouped as *Morphologic Lesions in Vitamin E Deficiency*, but they illustrate the interlocking of several disciplines. I think there is no field of inquiry which demands closer alliance between persons of different training and background. The biochemists occupy, and rightfully, the center of the stage—but anatomists, physiologists, nutrition workers, clinicians, veterinarians, and even pathologists have a finger in the pie and are in a position to contribute their mite to these all absorbing problems.

To one whose interests for many years have been focussed on human diseases, there is still—despite the intensive work on Vitamin E deficiency in many species of animals—a distressing lack of precise information as to the rôle of Vitamin E in human nutrition. What are the effects of tocopherol deficiency? Are there tissue changes comparable to those seen in laboratory animals, and are these sufficiently specific to serve as guiding signs to the pathologist? Have the newer biochemical methods for determination of tocopherol levels in blood, tissues, and food-stuffs made it possible to study the effects of tocopherol deficiency in man with greater precision? I am convinced that some of the papers included here provide at least a partial answer to these questions. • But more fundamental, of course, is the problem of the specific rôle of the tocopherols in cellular chemistry, and in this real progress is being made. A number of papers in this section add significantly to our knowledge of the part which the tocopherols play in enzymatic reactions and of how the normal processes are affected in their absence.

EFFECTS OF OVARIAN HORMONES UPON UTERINE PIGMENTATION IN VITAMIN E-DEFICIENT RATS*

By W. B. Atkinson,† H. Kaunitz and C. A. Slanetz‡

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Acid-fast pigmentation of the skeletal and cardiac musculature and the smooth muscle of the reproductive tract is now recognized as being characteristic of vitamin E deficiency in the rat. The chocolate-brown discoloration of the uterus was first noted in the E-deficient rat by Martin and Moore.¹ Subsequent histological studies demonstrated that pigment was deposited in fine granules in the cells of the uterine musculature.^{2, 3} Mason and Emmel⁴ further noted the presence of numerous pigment-laden macrophages scattered throughout the uterus of the E-deficient rat. Their findings indicated that the pigment was deposited first in the muscle cells and later transferred to the macrophages.

The rôle of the gonads in the regulation of the functional activity of the musculature of the reproductive tract, particularly that of the uterus, has suggested the possibility of a physiological relationship between the gonadal hormones and vitamin E. Mason and Emmel⁴ did not observe any diminution in muscle pigmentation in prepuberally ovariectomized animals as compared with intact controls. Ovariectomy, however, was followed by a decrease in the number of pigment-containing macrophages appearing during the course of the avitaminosis. More recently,⁵ it has been shown that a definite decrease in muscle pigment occurs when ovariectomized rats are maintained on a diet containing a lower percentage of fat than the ration used by Mason and Emmel.

The present experiments were undertaken to ascertain the effect of ovarian hormone treatment upon the deposition of uterine pigment in ovariectomized E-deficient rats maintained on a relatively low unsaturated fat intake.

Materials and Methods

Fifty-two female rats of the "Sherman" strain were used in these experiments. Most of the animals were born of mothers maintained on a vitamin-E-deficient simplified diet (TABLE 1) supplemented with 3 mg. of dl-alpha-tocopherol acetate per 100 gm. of ration.§ A few of the animals were born of mothers maintained on the deficient diet alone. The experimental rats were weaned at 3 to 4 weeks of age. The majority were immediately placed on the E-deficient diet. A few animals, however, were not placed on the diet until several weeks after weaning. The E-deficient ration limited the daily intake of alpha-tocopherol to approximately 30 μ g. per rat.

The young females were divided into several groups for subsequent study.

* Aided by grants from the Williams-Waterman Fund of the Research Corporation and the United States Public Health Service.

† Present address: Basic Sciences Division, Chemical Corps Biological Department, Camp Detrick, Frederick, Maryland.

‡ With the technical assistance of R. E. Johnson and A. Fuhr.

§ The alpha tocopherol acetate and other synthetic vitamins were supplied through the courtesy of Dr. Leo A. Pirk of Hoffman-La Roche, Inc.

Forty-one were bilaterally ovariectomized at 19 to 34 days of age. Weekly subcutaneous injections of the following substances were begun immediately: (a) 0.2 cc. of sesame oil—7 rats; (b) 5 μ g. of estradiol*—7 rats, 10 μ g. of estradiol—7 rats; (c) 5 μ g. of estradiol and 4 mg. of progesterone—2 rats, 10 μ g. of estradiol and 4 mg. of progesterone—9 rats; (d) 4 mg. of progesterone—9 rats. Crystalline hormones* were used, the weekly dose being dissolved in 0.2 cc. of sesame oil. The remaining 11 rats were not ovariectomized, but were injected with 0.2 cc. of sesame oil weekly. All animals were maintained on the E-deficient diet and the weekly injections were continued for a period of from 5 to 10 months, at which time the animals were sacrificed.

TABLE 1
COMPOSITION OF THE TOCOPHEROL-DEFICIENT DIET USED

<i>Basal Mixture</i>	<i>Per Cent</i>
Lard	10
Casein (Bordon's crude #453)	30
Cerelose	54
Celluration	2
Salt Mixture (Hawk Oser)	4
<i>Supplements to Basal Mixture</i>	<i>mg./kg.</i>
Thiamine Chloride	2
Riboflavin	4
Pyridoxine	4
Nicotinic Acid	100
Choline	1000
Vitamin K	4
p-amino-benzoic Acid	300
Ca Pantothenate	10
Oleum Percomorphum (ml./kg.)	0.2

The rats were autopsied promptly after sacrifice, and the uteri were fixed in Bouin's fluid. Tissue specimens were dehydrated in ethyl alcohol, cleared in xylene, and embedded in paraffin. Sections were cut 7 microns in thickness and were stained with hematoxylin and the Kinyoun modification of the Ziehl-Nelsen carbol fuchsin method to ascertain the presence and distribution of acid-fast pigment.

Observations

Histological examination of the uteri from the unsplayed rats treated with sesame oil alone revealed the presence of numerous strongly acid-fast granules throughout the cytoplasm of the myometrial cells. There was also a considerable number of pigment-containing macrophages scattered throughout the intramuscular connective tissue and, to a lesser extent, the endometrial stroma. No evidence of pigment deposition in the epithelial elements

* The alpha estradiol was supplied through the courtesy of Dr. Kenneth W. Thompson of Organon, Inc., the progesterone by Dr. F. E. Houghton of Ciba Pharmaceutical Products, Inc.

of the endometrium was discernible. These findings are characteristic of the intact E-deficient rat.

In the ovariectomized animals injected with sesame oil, on the other hand, there was complete absence of pigment in the longitudinal layer of the myometrium. The circular layer of muscle was peculiar in that the cells were filled with small granules which, unlike those in the intact animal, possessed but negligible to weak acid-fastness. In addition to these changes in muscle pigmentation, there was also a great reduction in the number of pigment-containing macrophages.

In 13 of the 16 animals treated with estrogen, acid-fast pigment was present in the same distribution seen in the uteri of the unoperated controls. The intensity of staining, however, was somewhat diminished. There was no discernible difference between the animals which had received 5 μ g. of estradiol and those which had received 10 μ g. of the hormone weekly. In 3 rats, there was but negligible pigment deposition. It is interesting to note that 2 of these animals had not been placed on the E-deficient diet until 2 to 3 weeks after weaning.

In the rats treated with progesterone, the amount and distribution of acid-fast pigment was not materially different from that in the castrates injected with sesame oil.

The results of treatment with estrogen and progesterone together are less clear-cut than in the preceding groups. In general, the distribution of pigment is similar to that seen in the animals receiving estrogen alone. However, in about half the animals receiving both hormones, there is a considerable reduction in the pigment of the longitudinal musculature and a decrease in the number of pigment-containing macrophages.

Discussion

The present observations clearly demonstrate that prepuberal ovariectomy results in a decreased accumulation of acid-fast pigment in the uterus of vitamin E-deficient rats maintained on a diet relatively low in unsaturated fats. Prolonged treatment with estrogen promotes uterine pigmentation in the ovariectomized animal, whereas progesterone alone does not have this effect. In fact, progesterone given concurrently with estrogen seems partially to neutralize the effect elicited by estrogen alone.

Evidence has been presented which indicates that the deposition of pigment in the E-deficient animal represents the peroxidation and polymerization of unsaturated fatty acids due to the decrease in tissue tocopherols which act as antioxidants.^{6, 7} Whether or not this represents the complete mechanism of pigment accumulation, it seems inescapable, from the present observations, that the effects of ovarian hormones on uterine pigmentation are mediated through their regulatory function in the metabolic processes of the tissues of the reproductive tract.

The well-known effects of the ovarian hormones on the morphology, contractility, and respiration of the myometrium indicate a profound metabolic influence. It may be surmised that the diminution of various physiological processes which accompanied ovariectomy is reflected in the decreased rate

of pigment formation. Conversely, the increased pigmentation in the uterus of the estrogen-treated castrate may be related to its concomitant increase in metabolic activity. Progesterone alone has but negligible effects on uterine activity and, under certain conditions, may act as an antagonist to estrogen. Here, again, the parallelism between the effects of hormonal influence on uterine metabolism and pigmentation is apparent.

An alternate explanation of the present observations might lie in the hypothesis that estrogen plays a direct rôle in the lipid metabolism of the myometrium. In any case, there is a paucity of information relating to the interaction of ovarian hormones and tocopherol in tissue metabolism. Further studies must be made before a definitive solution to the problem can be attained.

Summary

1. Rats ovariectomized at weaning and maintained 5 to 10 months on a vitamin E-deficient diet, relatively low in unsaturated fat, did not develop the acid-fast pigmentation of the uterine muscle characteristic of the intact E-deficient controls.

2. Ovariectomized rats treated with estrogen during the course of the avitaminosis developed uterine pigmentation similar to that seen in intact E-deficient animals.

3. Treatment of ovariectomized E-deficient rats with progesterone did not promote pigment deposition.

4. Progesterone given to E-deficient ovariectomized rats concurrently with estrogen may partially neutralize the pigmentation-promoting effect of the latter hormone.

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HISTOCHEMISTRY OF UTERINE PIGMENT IN VITAMIN E-DEFICIENT RATS*

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The pigment which develops in rats deficient in vitamin E not only serves as an index of the progress of the deficiency but may also provide a clue to the derangement of metabolism which leads to its production. The chemical characterization of the pigment is, consequently, of interest as a definition of an end point in metabolism. It is also necessary as a means of comparison between this pigment and others formed under different circumstances.

Many of the salient characteristics of the pigment of vitamin E deficiency have been determined by previous investigators. The methods of histological staining which have been used for its identification have also given information concerning its constitution. Attempts to extract the pigment from adipose tissue by Dam and Granados¹ and from uterus and skeletal muscle by Moore and Wang² have led to contradictory suggestions concerning its composition.

The present investigation has been restricted to the application of histochemical methods to tissue sections. Although these methods involve restrictions of temperature, solubility, and brutality of reagent, they have the unassailable advantage of guaranteeing that the observed reaction is due to the pigment itself and not to some artifact of extraction.

The investigations reported here were conducted entirely on the pigment present in the smooth muscle and associated macrophages of the uterus of rats on a vitamin E-deficient diet. Although there is reason to believe that this pigment is identical with that which develops in other organs in vitamin E deficiency, the term "uterine pigment" will be employed, since not all of the reactions reported here have been tested on the pigment of other organs. As a matter of convenience in this paper, it will be understood that the hemosiderin in the uterus is not included in the designation "uterine pigment."

Materials and Methods

The animals used in these experiments were from a highly inbred colony of Sherman albino rats which have been used in successive generations for the study of vitamin E deficiency for at least four years. They have been maintained on a vitamin E-deficient diet containing 10 per cent commercial lard. The tocopherol content was checked in cooperation with Doctors J. J. Beaver, Philip Harris, and Mary Quaife and was found to permit a daily intake of approximately 30 micrograms per adult rat. For the controls, the diet was completed by the addition of 3 mg. of synthetic dl-alpha-

* Aided by grants from the United States Public Health Service and the Williams-Waterman Fund of the Research Corporation.

† With the technical assistance of Éva Englander, R. E. Johnson, and A. Fuhr.

tocopherol acetate (Hoffmann-La Roche) for each 100 g. of diet, permitting a daily intake of roughly 300 micrograms per adult rat.

Uteri were available from 97 rats being utilized in another series of experiments. Due to the advantage of working with sections containing a large amount of pigment, the bulk of the reactions were first performed on the uteri of a rat 324 days old. The tissues were fixed in 10 per cent formalcalcium and, for comparison, in Bouin's fluid. All sections were cut 5 microns thick, either as frozen sections or after paraffin embedding. Embedding in paraffin did not alter the characteristics of the pigment.

Lipoid. The presence of lipoid in the uterine pigment is one of its most prominent characteristics. This can be most readily demonstrated by immersing either frozen or paraffin embedded sections in Sudan black B (FIGURE 1). Although any of the standard methods of applying this test will give equally positive results, the clearest differentiation is obtainable with the buffered solution of Sheehan and Storey.³ Since Sudan black B acts by differential solubility, accumulating in lipoids, it is a sensitive indicator of their presence. The fact that the pigment dissolves the Sudan in paraffin-embedded sections eliminates the possibility of confusion with ordinary fats, since they are removed by the alcohol and xylene treatment of the tissues preparatory to embedding.

Although Sudan black B can demonstrate the presence of lipoid, it cannot distinguish between the different members of this group. If cholesterol were present, it should give a positive reaction to the Liebermann test. However, after three days in a 2.5 per cent solution of iron alum at 37°C., the uterine pigment still did not give a positive response to concentrated sulfuric acid and acetic anhydride. It may be concluded, consequently, that the uterine pigment gives no evidence of the presence of cholesterol.

The possibility that acetal-phosphatides are present can be tested by the Feulgen plasmal reaction. When sections containing uterine pigment were immersed in saturated mercuric chloride for five minutes, followed by fuchsin sulfurous acid, they gave no indication of free aldehyde.

The insolubility of the pigment in the usual fat solvents indicates that the lipoid of the uterine pigment is not one of the ordinary fats or phospholipids. If the insolubility of the lipoid is not due to its presence in a lipoprotein combination, it would indicate that the lipoid is either extensively substituted or polymerized.

Protein. The possibility that the pigment may contain protein is of the utmost importance. The assumption that its insolubility in ordinary fat solvents necessitates a lipoprotein structure does not seem compelling, in view of the insolubility of polymerized fats. The extract prepared from vitamin E-deficient rats by Moore and Wang² provides more cogent evidence. Consequently, the following histochemical methods were applied to the uterine pigment.

Upon treatment with concentrated nitric acid, the pigment showed a slightly deeper yellow color than before treatment, while the reaction of the surrounding tissue was intense. Therefore, this xanthoproteic reaction might be interpreted as being positive, despite the interference of the natural

color of the pigment. By conducting a control experiment, however, doubt was cast on this conclusion. When oxidized cod-liver oil was subjected to the same test, a pronounced yellow color was obtained. Thus, the response of the pigment to concentrated nitric acid cannot be considered proof of the presence of protein.

Further tests for chemical groups which might be indicative of protein were applied. The details of the execution of these tests have been conveniently summarized by Serra.⁴ The biuret test was negative for the pigment but positive in surrounding tissue. Millon's reaction for phenolic groups and Voisenet's for indolic compounds were negative for the pigment but positive for other tissue elements. The nitroprusside reaction for sulfhydryl groups was negative both before and after treatment of the sections with potassium cyanide.

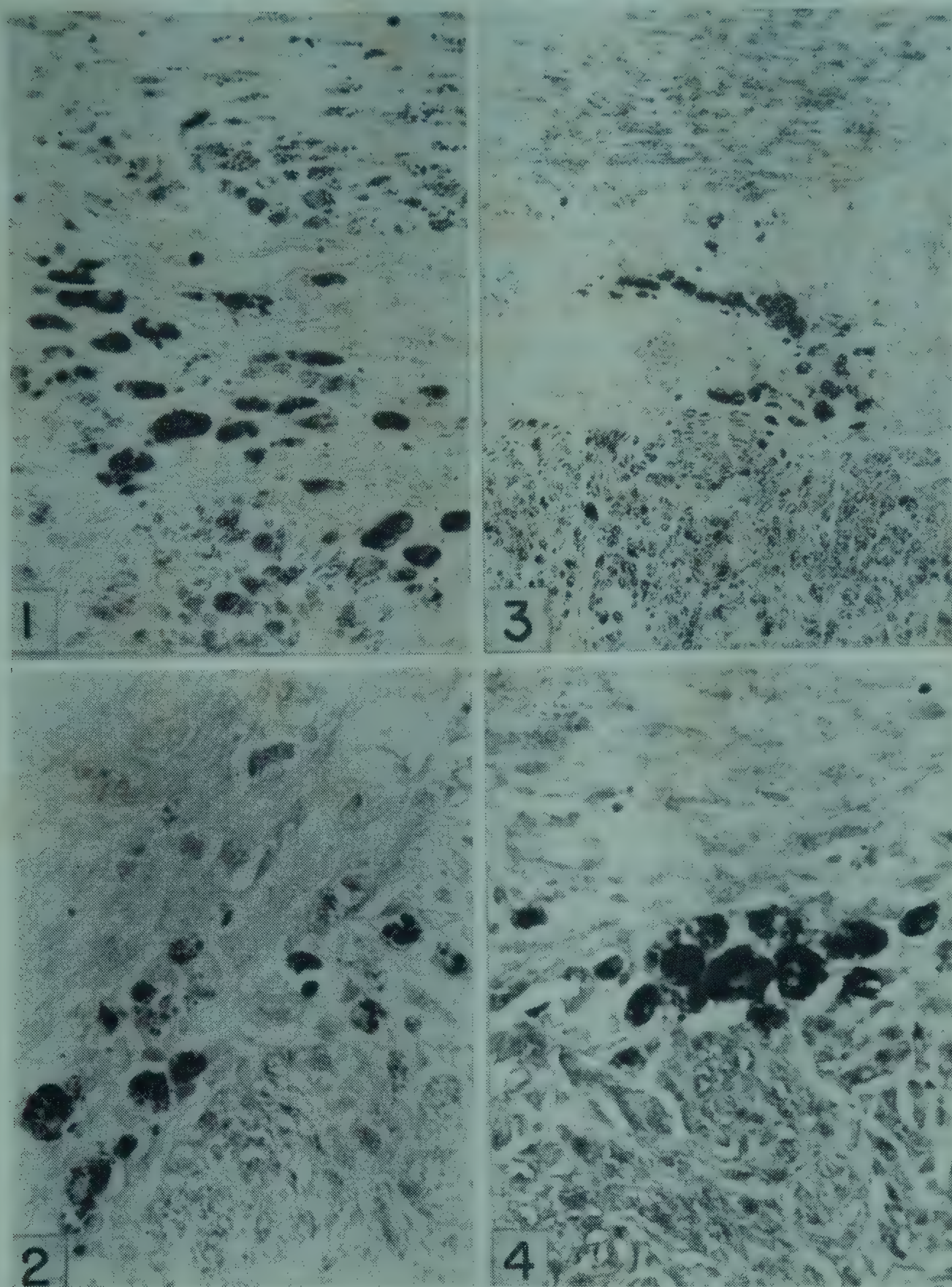
The net result of these tests may be summarized by stating that there is, at present, no direct histochemical evidence of the presence of protein in the uterine pigment. It would be unwarranted to assert definitely that protein is not present. Negative results may be due to interfering factors. The readiness with which the pigment reacts to other tests, however, allows some significance to be attributed to its lack of response to tests for protein.

Carbohydrate. Although there has been no suggestion that the pigment contains carbohydrate, the possibility was investigated. After oxidation with 1 per cent periodic acid for 20 minutes, the pigment becomes intensely red when immersed in fuchsin sulfurous acid. The reaction is equally brilliant, however, when the tissues are digested with saliva before periodic oxidation. Consequently, glycogen is not responsible for this reaction. The significance of the periodic acid oxidation will be considered with other oxidation reactions.

The absence of sulfuric acid esters of mucopolysaccharids from the pigment is indicated by the lack of metachromatic staining with toluidin blue. The color of the pigment after staining with this dye is similar to that of the nuclei and contrasts markedly with the metachromatic staining of the mast cell granules.

Dissociation. The affinity of the uterine pigment for basic dyes (FIGURE 2) has long been used in staining methods for its visualization. The possibility of using the degree of basophilic exhibited at different degrees of acidity as an index of the dissociation of the substance was pointed out by Pischinger⁵ and has been widely used on other histological objects. Sections of the uterus were incubated for 1 hour at 37°C. in 0.01 per cent methylene blue, with the pH ranging from 0 to 9. Michaelis barbitol-acetate buffer was employed, except for pH 0 and pH 1, which were accomplished with unbuffered HCl, and pH 2, for which HCl-KCl buffer was used. Under these circumstances, the pigment does not stain at all below pH 3 and, at that pH, only extremely faintly. At pH 4, the pigment stains distinctly, but the intensity of color increases with further decrease in acidity. At pH 4, the nuclei stain more intensely than do the pigment granules, but this situation is reversed under less acid conditions.

The significance of these observations might be elaborated unduly by



FIGURES 1-4

Photomicrographs of the uterus of a vitamin E-deficient rat. The sections were cut 5 microns thick from paraffin-embedded tissue. The photographs are oriented with longitudinal musculature below and circular musculature above, with pigment-laden macrophages between the two layers of muscle.

FIGURE 1. ($\times 185$) Bouin fixation, Sudan black B stain. The affinity for Sudan black B demonstrated by the pigment in the muscle fibers and in the macrophages shows the lipoid nature of the pigment.

FIGURE 2. ($\times 185$) Formal-calcium fixation, crystal violet-methyl green stain. The pigment is deeply stained by the crystal violet, contrasting with the methyl green staining of the nuclei.

FIGURE 3. ($\times 100$) Formal-calcium fixation, oxidation by Foot's diammine silver carbonate for 24 hours at room temperature. The reduced silver shows the location of the pigment and gives an indication of its oxidation potential.

FIGURE 4. ($\times 185$) Formal-calcium fixation, periodic acid oxidation followed by fuchsin sulfurous acid. In the absence of glycogen, polysaccharids, and protein, this response to periodic acid oxidation is probably due to derivatives of unsaturated fat.

detailed comparison of the dissociation of the pigment with that of the other tissue constituents. The contrast with the mast cell granules is, however, particularly striking. Even at pH 0, these granules stain deeply with

methylene blue. The range of response of the pigment to varying acidity bears a superficial resemblance to that of ribonucleic acid. Incubation of sections for 2 hours at 57°C. in a solution containing 0.5 mg. of ribonuclease in 100 ml. and buffered to pH 6.8 with McIlvaine's buffer did not alter the staining reaction of the pigment. There is, consequently, no indication of the presence of ribonucleic acid.

The possibility that the acid dissociation of the pigment may be associated with the lipid remains for consideration. Until the nature of the lipid is fully determined, a definitive answer to this question cannot be given, but its probability is strongly indicated by the following experiment. Cod-liver oil was allowed to oxidize by being exposed to air in a thin film for a few days at elevated room temperature. The oxidized oil responded to methylene blue staining at varying pH in the same manner as the pigment, in addition to resembling it in its reactions to the other tests.

Of the vast array of basic dyes which might be employed for staining the pigment, members of the triphenylmethane series have been most popular. We have used basic fuchsin, crystal violet, and methyl green, named in the order of increasing methylation. For routine staining, we have found Nicolle's carbol crystal violet diluted with 20 parts of water to stain adequately in one and one half minutes. Momentary immersion in 0.01 N HCl leaves the pigment well-stained, and mounting can be done conveniently in Apathy's mixture. Definition of the pigment is increased by viewing the slide with a Wratten G filter in the optical system. The color of the crystal violet stain of the pigment is not altered by treatment with Lugol's solution, indicating that the pigment is not Gram positive.

When tissue sections are placed for an hour in 10 per cent ferric chloride dissolved in normal acetic acid, subsequent treatment with ferrocyanide results in Prussian blue coloring in both pigment and nuclei. This affinity of the pigment for ferric ions is to be expected from its dissociation and is of interest in connection with the staining of the pigment by iron hematoxylin.

Oxidation. The oxidation potential of the pigment is an important identifying characteristic. Of greatest general utility has been oxidation by silver diammine, since the reduction of the silver results in deposition of the metal. Using Foot's diammine silver carbonate in 70 per cent alcohol, the uterine pigment showed some reduction of silver after 20 minutes at 57°C. After 24 hours at room temperature the marked reduction shown in FIGURE 3 was achieved. More rapid reduction can be obtained with other silver diammine solutions, but the results with Foot's reagent are valuable for comparison with other pigments.

Another useful reagent is a mixture of equal parts of 1 per cent ferric chloride and 1 per cent potassium ferricyanide. With the reduction of either component, a bluish precipitate is formed. The uterine pigment is able to accomplish this reduction in five minutes after paraffin embedding.

As would be expected from the oxidation reactions described, it is also possible to oxidize the pigment with permanganate and with molybdic acid. The immersion of sections in 0.4 per cent potassium permanganate dissolved

in 0.12 per cent potassium hydroxide for 30 minutes at 5°C. results in brown coloring of the pigment by manganese dioxide. Under these conditions, unsaturated fatty acids will reduce the permanganate, but, needless to say, this test is not specific for such a configuration.

Oxidation by means of periodic acid is capable of providing more precise information concerning the structure of the pigment. After 20 minutes in 1 per cent periodic acid, the pigment stains intensely with fuchsin sulfurous acid (FIGURE 4). Of all the reactions which we have tried on the pigment, this appears to be the most sensitive one for its visualization.

Since oxidation by periodic acid is indicative of the presence of reactive groups, such as hydroxyl, keto, or amino groups, on adjacent carbon atoms, it would be expected to be effective on the derivatives of unsaturated fatty acids formed after initial peroxidation. Since glycogen and acid polysaccharids are believed to be absent from the pigment, on the basis of evidence given earlier, it seems probable that the results of oxidation of the pigment by periodic acid are due to properties of the lipoid. This probability is increased by the similarity in the response of the pigment to that obtained in experiments with periodic acid on oxidized cod liver oil.

Iron. Since the presence of iron is one of the key characteristics in the classification of pigments, exhaustive tests for iron were conducted. A positive reaction on the part of the hemosiderin present in the macrophages of the endometrium was a useful indication of the effectiveness of the methods. In a similar fashion, the absence of iron contaminants in the reagents was checked by noting the absence of an iron reaction in the nuclei.

Efforts were made to unmask iron by incubating in 3 per cent nitric acid in 95 per cent alcohol for 36 hours at 37°C., with subsequent application of 1½ per cent potassium ferrocyanide mixed with an equal volume of ½ per cent hydrochloric acid. Hemosiderin showed the Prussian blue color but the uterine pigment gave a negative reaction. After immersion in potassium ferrocyanide, sections were subjected to hydrochloric acid vapor. Again the hemosiderin was positive and the uterine pigment negative. Reduction in ammonium sulfide resulted in brown iron sulfide coloring in the hemosiderin, and this produced Turnbull's blue upon subsequent application of ferricyanide. But the uterine pigment was negative under both circumstances.

Discussion

When the results of the present investigation are reviewed, it becomes evident that all of the positive reactions obtained could be produced by a lipoid resulting from the polymerization of oxidized unsaturated fat. The possibility of the transformation of fat into pigment has been studied by several investigators. The work of Hass⁶ focused attention on the polymerization of peroxides of unsaturated fats, and Endicott⁷ suggested this origin for ceroid. Dam and Granados¹ applied this theory to the pigment developed in the adipose tissue of vitamin E-deficient rats. They demonstrated the presence of peroxide in the extracted pigment by means of potassium iodide. Granados *et al.*⁸ developed a histochemical test for peroxide and applied it to sections of adipose tissue. In view of the usually transi-

tory nature of the peroxides or hydro-peroxides of fats, it may well be that the end-products can be more effectually studied by periodic acid oxidation.

Although the present research has provided no evidence of protein reactions on the part of the pigment, it would not be warranted to conclude that the absence of protein has been proven. The pigment extracted by Moore and Wang² strongly suggests a protein origin. The identity of any extract with the substance sought must be subject to rigid proof. Although yellow fluorescence was well established by Moore and Wang⁹ as a significant characteristic of the pigment of vitamin E deficiency, it does not define it uniquely. It is a valuable criterion when employed in conjunction with the others which are summarized in this paper.

The relation of the pigment of vitamin D deficiency to other pigments can be answered only partially at the present time. The absence of demonstrable iron, the presence of lipoid which is insoluble in fat solvents, and the strong affinity for basic dyes qualify the pigment of vitamin E deficiency for inclusion in the group of lipofuscins. Its relationship to the various pigments which belong to this group is more difficult to establish.

The similarity of the pigment of vitamin E deficiency to ceroid has been pointed out by Mason and Emmel,¹⁰ Dam and Granados,¹ Victor and Pappenheimer,¹¹ and Pappenheimer and Victor.¹² The term "ceroid" was introduced into pigment nomenclature by Lillie *et al.*¹³ for a particular lipofuscin associated with hepatic cirrhosis in rats fed low-protein diets. Ceroid was more fully characterized by Endicott and Lillie¹⁴ by means of an extensive series of histochemical tests. From these published descriptions of ceroid, the uterine pigment of vitamin E deficiency differs in the readiness with which it reduces both Foot's ammoniacal silver carbonate solution and the ferric chloride-ferricyanide reagent. In view of the long history of controversy which has attended the application of reduction reactions to the characterization of pigment, as reviewed by König,¹⁵ it may well be that further investigation of ceroid will show that it is identical with the pigment of vitamin E deficiency.

Summary

The pigment of vitamin E-deficient rats, as studied in the uterus, gives the following characteristic reactions:

1. Strong affinity for Sudan black B.
2. Combination with basic dyes at pH 4 and above.
3. Reduction of silver diammine and ferric chloride-ferricyanide.
4. Oxidation by periodic acid.
5. Insolubility in ordinary fat solvents.
6. Absence of demonstrable iron.

All of the characteristic reactions of the pigment are also given by oxidized cod-liver oil. Since histochemical tests for protein were uniformly negative, the present investigation adds evidence in favor of the origin of the pigment of vitamin E deficiency by the peroxidation and polymerization of unsaturated fat.

On the basis of these reactions the pigment may be classified as a lipo-

fuscine. Although it differs from the published descriptions of ceroid in its oxidation potential, further study of ceroid may establish a fundamental similarity.

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Discussion of the Papers

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): It seems quite likely, though it has not been established by critical test, that the pigment arising in tissues of the vitamin E-deficient animal is identical to the so-called "ceroid" pigment of nutritional liver cirrhosis. It should be kept in mind, however, that the latter may represent, for the most part, the oxidation products of fats abnormally mobilized and stored in liver cells, while the former pigment may constitute oxidation products of intracellular lipids related to the normal functioning of smooth, skeletal and cardiac muscle fibers. Certainly, the occurrence of either pigment can be prevented by adequate administration of vitamin E.

Dr. H. ELFTMAN (*Department of Anatomy, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): The general term lipofuscin is applicable to both ceroid and the acid-fast pigment of vitamin E deficiency. The possibility that the two pigments are identical in spite of recorded differences in their oxidation potentials can only be answered by the application of more specific tests to ceroid than those usually used for its identification.

Iron-hematoxylin staining was not employed in the present investigation, but Dr. Mason's question can be answered from other experiments. Mor-danting with ferric iron resulted in combination of the iron with the pigment, as demonstrated by the blue color produced by subsequent treatment with ferrocyanide. Hematoxylin would also combine with this iron.

It is to be hoped that Dr. Moore will persist in his efforts to extract the pigment. Increased assurance that the extract is comparable to the native pigment can be obtained by testing not only for fluorescence but also for the other reactions which the pigment gives in tissue sections.

RESTORATION OF VAGINAL ESTRUS BY ALPHA-TOCOPHEROL ACETATE IN OLD RATS*

By S. Y. P'an, H. Kaunitz, and C. A. Slanetz†

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It has been reported by Slonaker¹ that lengthening of the estrous cycle occurs in older rats on normal diet. Vlcek² has noted an earlier onset of irregularities of the estrous cycle in vitamin E-deficient rats. Kaunitz and Slanetz³ have observed that the pregnancy rate of rats maintained on a diet with "normal" tocopherol content declines 50 per cent when the rats are over one year of age. From their experiments with additional tocopherol supplements in E-deficient animals, they,⁴ as well as Emerson and Evans,⁵ concluded that the vitamin E requirement of rats increases with age.

This report deals with the question as to whether the administration of alpha-tocopherol is able to shorten the prolonged estrous cycle in old rats on rations with tocopherol content equivalent to that in most laboratory diets.

Methods

Sixty-eight albino rats of a highly inbred stock, 8 to 24 months in age and maintained for successive generations on a simplified diet‡ containing 3 mg. of synthetic dl-alpha-tocopherol acetate§ per 100 gm., were employed for this experiment. The tocopherol content of the diet approximated that of the usual laboratory rations. Vaginal smears from these rats were examined daily, except on Sundays, for at least two weeks and generally for 4 to 6 weeks. To 17 female rats, the estrous cycles of which averaged more than 18 days, 30 to 60 mg. of dl-alpha-tocopherol were given once a week orally for 3 to 8 weeks. Eight rats with cycles of 10 days or more were left untreated during the same period as those under treatment in order to serve as controls to rule out spontaneous shortening of the estrous cycle.

Results

In view of the fact that vaginal smears were examined only 6 days a week, we thought it justifiable to accept 7 days as the upper limit of a normal estrous cycle. An analysis of the estrous cycles in the 68 rats is given in

* Aided by grants from E. R. Squibb and Sons, the U. S. Public Health Service, and the Williams-Waterman Fund of the Research Corporation.

† With the technical assistance of Adelheid Fuhr, Ruth Ellen Johnson and Elaine T. Mackenzie.

‡ The diet consisted of:

	Parts		mg./kilo
Commercial lard	10	Pyridoxine	4
Crude casein	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Cellu-ration	2	Choline	1000
Salt mixture	4	Vitamin K	4
		p-aminobenzoic acid	300
		Calcium pantothenate	10
		Oleum percomorphum	200
		dl-alpha-tocopherol acetate	30

§ Dr. Leo A. Pirk of Hoffmann-La Roche, Inc. kindly supplied us with synthetic dl-alpha-tocopherol acetate.

TABLE 1, in which the percentage of rats with normal cycles is tabulated according to the age of the rats. It can be seen from the table that the number of rats with normal cycles decreases with increase in age. Statistically, a significantly greater number of rats over 300 days in age have prolonged estrous cycles than those younger than 300 days in age ($\chi^2 = 6.6$).

TABLE 2 summarizes the effect of additional weekly doses of 30 to 60 mg. of tocopherol on the prolonged estrous cycle of older rats. In 9 out of 17 rats thus treated, the cycle became normal within 3 to 8 weeks. In the 8 controls that received no additional tocopherol, no noticeable change

TABLE 1

PERCENTAGE OF RATS OF DIFFERENT AGE GROUPS WITH ESTROUS CYCLES OF SEVEN DAYS OR LESS

<i>Age of rats in days</i>	<i>Number of rats</i>	<i>Average estrous cycle 7 days or less</i>
		%
250-300	11	73
301-500	36	36
501-700	21	24

TABLE 2

EFFECT OF THE ADMINISTRATION OF ALPHA-TOCOPHEROL ON THE LENGTH OF ESTROUS CYCLES OF OLD RATS

<i>Before treatment</i>				<i>After treatment</i>		
<i>No. of rats</i>	<i>Average age in days</i>	<i>Total duration of observations (range in days)</i>	<i>Average days/cycle</i>	<i>Average age in days</i>	<i>Total duration of observations (range in days)</i>	<i>Average days/cycle</i>
3	376.6	24-41	18.3	424	21-22	5.6
6	450	14-27	>18.0	468.5	22-56	5.7
4	404.5	14-30	21	429	21-57	32
4	494	14-30	>19.2	512	21-36	24.5
8*	455.7	18-34	14.1*	510.5	19-28	14.7*

* Control rats which received no alpha-tocopherol.

in the length of the estrous cycle was ever noted. Statistically, the length of the estrous cycles are shortened in a significant number of rats that have received additional doses of alpha-tocopherol ($\chi^2 = 6.0$).

Discussion

These results indicate that the administration of alpha-tocopherol was capable of restoring the prolonged estrous cycle to normal in a significant number of older female rats maintained on a diet with the conventional tocopherol content. It seems, also, that the onset of lengthening of the estrous cycle in rats depends a good deal on the amount of tocopherol present in the diet; because, from the examination of the estrous cycle in 13 rats on

vitamin E-deficient diet, we found that in none of them did the estrous cycles average less than 7 days in duration when the rats had an average age of approximately 300 days, while a relatively high percentage of rats of this age group on the "normal" diet showed normal cycles.

Summary

1. The length of the estrous cycle was examined in 68 rats of from 8 to 24 months, maintained on a simplified diet permitting a daily intake of about 300 micrograms of dl-alpha-tocopherol acetate, which approximated the intake of ordinary laboratory rats.

2. The number of rats above one year in age with lengthened vaginal estrus is significantly higher than that of younger animals.

3. The administration of 30 to 60 mg. of alpha-tocopherol acetate weekly to 17 rats with definitely lengthened estrus restored the cycle to normal in 9 females. No spontaneous restoration was noticed in 8 control rats.

4. It was concluded that tocopherol deficiency is one of the factors responsible for the prolongation of the estrous cycle in rats on diets with the conventional tocopherol content.

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INCREASED TOCOPHEROL REQUIREMENTS DURING THE RAT'S MENOPAUSE*

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In the majority of old, female rats, there develops a syndrome consisting of gradual disappearance of the vaginal estrous cycle and sterility. This has previously been termed "menopause."¹ These studies deal with the question as to whether tocopherol deficiency plays a part in the menopausal syndrome of the rat and, beyond this, whether tocopherol is involved more generally in processes of "aging."

The experiments were carried out on a highly inbred colony of albino rats maintained on the diet shown in TABLE 1. The basic tocopherol content of this diet, which was determined in cooperation with Beaver,² Harris, and Quaife, permitted a daily intake of roughly 30 micrograms of alpha-tocopherol per adult rat. However, most of the rats used in these studies were given a supplement of 3 mgs. synthetic dl-alpha-tocopherol acetate† per 100 gms. of diet, permitting a daily intake of 300 micrograms. This approximates the conventional tocopherol content of many laboratory diets.

In the experiments concerned with the ability of the rat to become pregnant, nearly 800 rats were used within a period of four years. Many of the females were used repeatedly. They were mated either 3 to 8 weeks after a pregnancy or within 4 weeks of a previous negative mating. The females were left with a fertile male for a period of five days during the first part of our experiments and for two weeks in the second part of the studies, when most of the tests on old rats were done. Two weeks after mating had begun, the animals were tested repeatedly for the placental‡ sign and weighed almost daily. A resorption gestation was recorded if a positive placental sign was followed by weight increase and gradual weight loss. In the absence of the pregnancy sign and weight differences, it was concluded that the rat was not pregnant. Laparotomy was done in more than a hundred instances in which the results had not been clear cut. In more than 90 per cent of such doubtful cases, the result of the laparotomy coincided with the tentative "clinical" diagnosis. We are confident, therefore, that the number of errors committed is negligible.

In previous studies (FIGURE 1) of the pregnancy rate in normal and E-deficient rats,^{3, 4} we found that it increased gradually among animals on the complete diet to about 85 per cent when they were 3-4 months old, which is in agreement with the observations of Evans and Burr⁵ and Goettsch and Pappenheimer⁶ on the pregnancy rate in a normal rat colony. After the fourth month, the pregnancy rate of the animals on the complete diet de-

* Aided by grants from the U. S. Public Health Service and the Williams-Waterman Fund of the Research Corporation.

† Dr. Leo A. Pirk of Hoffmann-La Roche, Inc. kindly provided us with synthetic dl-alpha-tocopherol acetate and most of the other vitamins.

‡ 13-15 days after a positive mating, blood appears in the rat's vagina indicating the formation of placental tissue.

TABLE 1
COMPOSITION OF VITAMIN E-DEFICIENT DIET

Basal mixture		Supplements of basal mixture	
	%		mg./kilo
Casein, crude	30	Thiamine chloride	2
Cerelose	54	Riboflavin	4
Lard, commercial	10	Pyridoxine	4
Salt mixture (Hawk-Oser)*	4	Calcium pantothenate	10
Celluration	2	p-Amino benzoic acid	300
		Choline	1000
		Inositol	1000
		Vitamin K	4
		Oleum percomorphum†	200

* HAWK, P. B., B. OSER, & W. H. SUMMERSON. 1947. Practical Physiological Chemistry. 12th Edition: 1273. The Blakiston Co. Philadelphia.
† During the last months of the experiments, oleum percomorphum was replaced by 3 mg. crystalline beta carotene and 10 micrograms crystalline vitamin D₂ (Calciferol) per 1000 grams of diet. We are indebted to Dr. Harold M. Barnett of the Barnett Laboratories for the carotene and to Dr. M. L. Tainter of the Sterling-Winthrop Research Institute for the Calciferol.

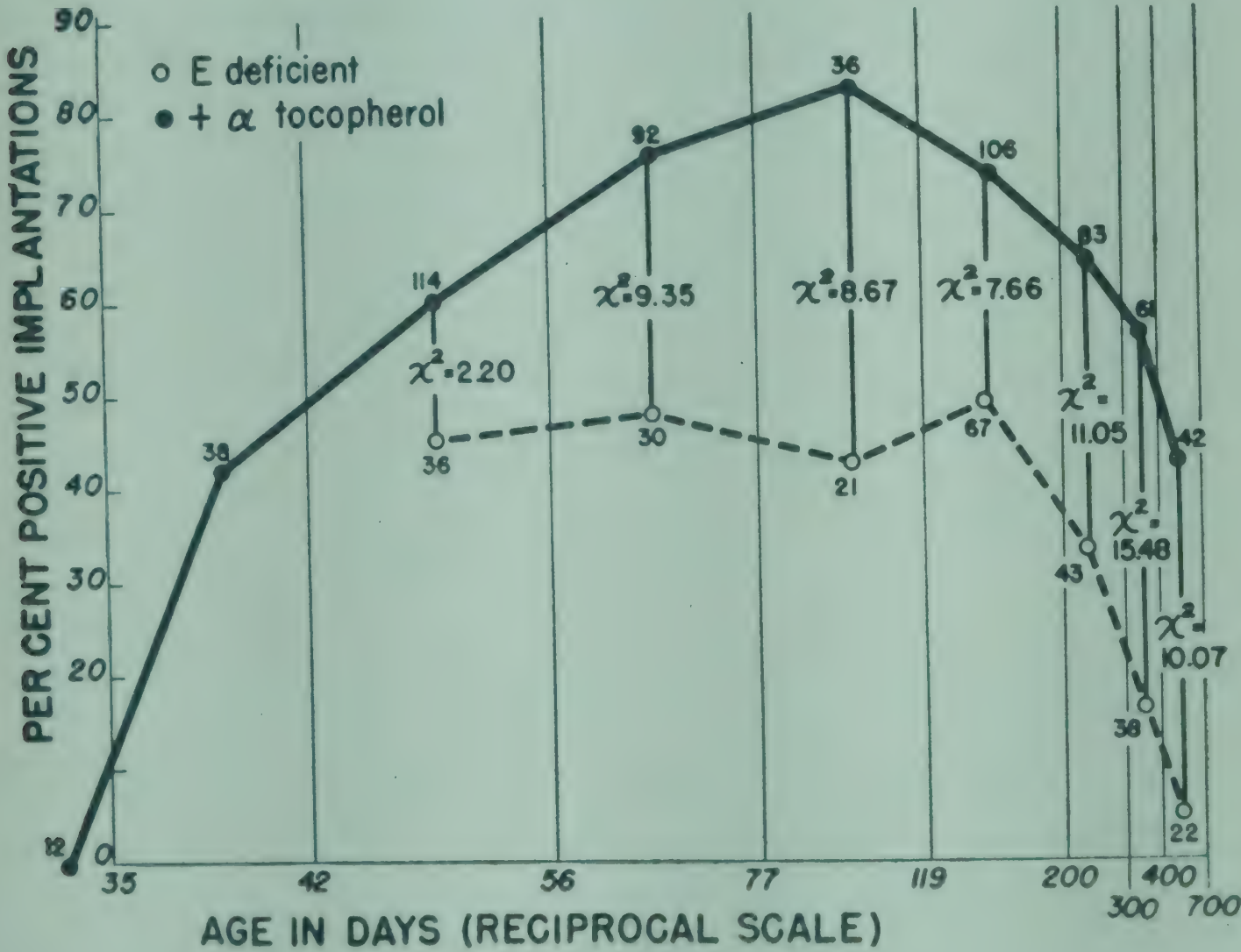


FIGURE 1.

clined steadily. At one to one and a half years, it was roughly 50 per cent. In the E-deficient group, the pregnancy rate after 8 weeks was significantly lower than that of the control group and continued to be so throughout life. At 3 to 5 months, it was about 50 per cent, and it declined sharply thereafter. Our main interest was now concerned with the question as to whether tocopherol deficiency played a part in the low pregnancy rate of the old

animals on the complete diet or whether their sterility was due to other factors.

The females on the complete diet which had proved to be sterile on at least three consecutive five-day or two fourteen-day matings were divided into two groups. One group was given a weekly oral supplement of 30-60 mgs. alpha-tocopherol acetate per rat, and they were mated again. The other group continued to be repeatedly mated without additional alpha-tocopherol supplements. TABLE 2 demonstrates the results of these experiments. Nearly half of the animals with additional tocopherol became pregnant again. Although some of the controls also became pregnant again, the

TABLE 2
PREGNANCIES IN OLD RATS AFTER REPEATED STERILE MATINGS

	<i>No. of experiments</i>	<i>% Preg- nancies</i>	<i>% Sterile</i>
30-60 mgs. tocopherol supplement	46	43.5	56.5
"Normal" tocopherol supplement	38	16.0	84.0

Massive tocopherol supplements to "normal" diet increase the pregnancy rate significantly.

TABLE 3
PREGNANCY RATE IN "STERILE" RATS AFTER MASSIVE TOCOPHEROL SUPPLEMENTS
ACCORDING TO THE AGE AT ONSET OF "STERILITY"

	<i>No. of experiments</i>	<i>% Preg- nancies</i>	<i>% Sterile</i>
Onset of "sterility" before 200 days	14	71.5	18.5
Onset of "sterility" after 200 days	32	31.0	69.0

Treatment of younger rats leads to higher pregnancy rate than in old animals.

difference in the pregnancy rates is highly significant. It is probable that the pregnancy rate would have been approximately 60 per cent if we could have excluded the rats with infected uteri. They number about twenty-five per cent in this age group.

In TABLE 3, the rats with additional tocopherol supplements are divided into two groups, according to their age at onset of sterility. The latter was taken to be the age at which the first negative mating occurred. The table demonstrates that the animals were more susceptible to tocopherol treatment if the onset of sterility occurred before the 200th day. Inasmuch as about 150 days elapsed after the onset of sterility in both groups of TABLE 3 before tocopherol administration started, the experiment demonstrates that the changes leading to sterility can more easily be counteracted by tocopherol if its administration starts at an earlier age.

In previous experiments, we found, in agreement with Emerson and Evans,⁷ that the tocopherol requirements of rats maintained on vitamin E-deficient diets increase steeply with age. This is also the case when the rats are maintained throughout life on a diet with a relatively high tocopherol content. These experiments permit a rough calculation of the tocopherol requirements of rats of various ages.

Rats maintained on a diet permitting a daily intake of 30 micrograms had a pregnancy rate of 50 per cent at 3 to 5 months. Those on a diet with a daily intake of 300 micrograms had the same pregnancy rate at 1 to 2 years. Inasmuch as tocopherol deficiency could be proved to be an important factor in the low pregnancy rate of the two groups and both groups had the same pregnancy rate, it can be concluded that the tocopherol requirements of rats increase roughly tenfold from the first half year of life to the second year.

It must be emphasized, however, that tocopherol is only one of the factors involved in the old rats' sterility; because we noted that all the rats eventually became sterile, despite continued tocopherol treatment.

The question of whether the ovary or the uterus is more responsible for the rat's menopause is of considerable interest. In experiments carried out with Blandau⁸ of Dr. Karl E. Mason's laboratory, it was found that the production of ova and their fertilization and transport through the oviduct did not differ in old, vitamin E-deficient females and females of the same age group on the complete diet. This was in agreement with experiments in which it had been observed that post-mating administration of tocopherol to vitamin E-deficient females increased the pregnancy rate significantly. These results would tend to make the uterus, rather than the ovaries, responsible for the low pregnancy rate in vitamin E deficiency. It seems that the implantation of the ovum in the uterus—in other words, the earliest stages of uterine pregnancy—are disturbed.

Further evidence for uterine, rather than ovarian, dysfunction was found in experiments with Dr. P'An of Dr. Van Dyke's laboratory, demonstrating that the implantation of the ovary of an old, vitamin E-deficient rat into a young, spayed rat produces vaginal estrus in the same fashion as the ovaries of normal animals.

In experiments carried out with Atkinson,⁹ it was noted that the formation of uterine pigment was reduced after castration and could be provoked by injection of estradiol. This could indicate that the uterine pigmentation is a consequence of the sex hormone stimulus provided by the normally functioning ovaries.

It seems very probable at present, therefore, that the reproductive disturbances of the rat in vitamin E deficiency are due to uterine, rather than ovarian, changes. This uterine dysfunction is accompanied by the presence of fibrosis and pigment. The latter, as the experiments with Elftman¹⁰ have brought out, belongs to the group of "Abnützungspigments." Both fibrosis and pigmentation of the sex organs are a manifestation of aging.¹¹

Changes associated with senescence were also found in studies of the life-span of 386 rats. It was observed that, in both males and females, the

average lifetime of the deficient group was significantly shorter than that of the controls. Weight deficits, also often an expression of aging, are pronounced in the deficient animals. These differences become noticeable after the third to fourth month,¹² particularly if the weights are plotted according to Zucker and Zucker.¹³

Skin lesions of a nonspecific character—another frequent sign of aging—are seen among the deficient animals at a much earlier age than among the controls. If one adds the early onset of the menopause, the occurrence of resorptions, the decreased pregnancy rate, the formation of uterine fibrosis and “Abnützungspigment,” and the loss of male fertility, all of which are symptoms of aging, it becomes evident that tocopherol deficiency is deeply involved in the processes which accompany aging. How specific this influence is or how far it could be the nonspecific expression of any chronic deficiency remains to be seen. One wonders, however, whether the effect of vitamin E deficiency on aging indicates that aging is a consequence of various deficiency states, rather than the result of “natural” processes necessarily inherent in the organism.

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THE BIOLOGICAL ASSAY OF VITAMIN E BY THE "MALE RAT TEST"

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Introduction

In 1940, K. E. Mason found that the minimum dose of alpha-tocopherol necessary to prevent testicular atrophy in the male rat is between 35 and 70 gammas per day. At the same time, in a paper presented at the Second Pan American Congress of Endocrinology in Montevideo in 1941, we showed that 30 gammas of alpha-tocopherol, given daily during a period of six months, prevented testicular atrophy in the white rat, enabling these animals, in 100 per cent of the cases, to impregnate normal females.

With the purpose of determining the possibility of using testicular atrophy in the male rat as a quantitative criterion for the biological determination of vitamin E, a series of experiments was undertaken based on the fact that it is possible to prevent testicular atrophy in the rat by administering a small dose of alpha-tocopherol and also on the fact that when the amount of vitamin E received does not cover the animals' requirements they suffer testicular atrophy. Thus, the percentage of sick animals was found to be greater the smaller the amount of vitamin E intake.

Experimental Part

Animals Used. White male rats were used. The strain of the animals of our laboratory descends from rats sent, about 15 years ago, by the Wistar Institute of Philadelphia. The mothers received the same food as other animals in the breeding colony until the offsprings were 13 days old. They were then given a diet lacking vitamin E. Each female had procreated six male litter mates, and one was placed in each lot. In this manner, the reserves of vitamin of the animals in all lots were equal and were reduced to those received from the mother through the placenta and in the milk. The animals were taken away from their mothers and placed in cages with double bottom on the 24th day.

The diet

Casein (vitamin-free).....	200
Wheat starch.....	700
Salts (O.M.).....	40
Fresh yeast (beer).....	50
Cod-liver oil.....	10

This diet was administered *ad libitum*.

Administration of Supplements. The supplements were administered daily orally in olive-oil solution. This was initiated when the animals were 30 days old in the first and second experiments and when they were 40 days old in the third experiment. The administration of supplements was continued until the death of the animals, which occurred when they were approximately 100 days old.

Criterion Used for Determining the State of the Testicle. While the animal was alive, the criterion used to determine the state of the testicle was external palpation. Three degrees were established, which correspond to one, two, or three crosses (+ ++ +++):

- + Means a slight reduction in size and consistency of the testicle.
- ++ Means approximately a size half the normal.
- +++ Means a great reduction in size and consistency, which makes the testicle difficult to find by palpation.

During the course of the experiment, the animals were inspected once a week until one of them was found to have grade +, and from then on, every three days. The litter mates were sacrificed when the one receiving no supplement presented testicular atrophy +++ during the last ten days. One of the testicles was kept in a solution of 10 per cent formol, put in paraffin and studied histologically. The other testicle was weighed immediately after being taken out and dried afterwards at a temperature of 50°C. until the weight was found to be constant.

TABLE 1

Lot no.	Daily supplement (six months)	Testis weight fresh	Testis weight dried	Results: histological examination (Mason scale)					
1	600γ tocopherol	1.62	0.22	N-	N	N	N	N	N
2	300γ tocopherol	1.65	0.24	N	N	N	N	N	N
3	60γ tocopherol	1.64	0.23	N	N	N	N	N	N
4	30γ tocopherol	1.72	0.25	N-1	N	N	N-1	N	N
5	15γ tocopherol	1.25	0.18	5	1	1	5	5	5
6	6γ tocopherol	0.69	0.06	5	5	5	5	5	5
7	0γ tocopherol	0.71	0.08	5	5	5	5	5	5

Investigations Made. In the first investigation, made in 1940, the minimum dose of alpha-tocopherol necessary to prevent testicular atrophy in the white rat was determined. The results showed that 30 gammas of tocopherol, when administered daily during six months, prevented testicular atrophy (TABLE 1).

The second investigation was made to determine whether the animals of the same breed suffer testicular atrophy in the same period. Eight groups of litter mates were used, and the results indicated that, except in the case of one animal, the time of onset of testicular damage did not vary more than ten days and, once initiated, degeneration was completed very rapidly.

The third investigation was made in twelve lots of six litter mates. The work was divided into two experiments, made with the purpose of determining if the value obtained in the chemical titration of the non-saponifiable fraction of wheat germ and of wheat-germ oil was in proportion with the capacity of these materials to prevent testicular atrophy in the rat (TABLES 2 and 3).

In these experiments, twelve lots of six rats each were established. These lots received, in addition to the basal diet lacking vitamin E, the following supplements:

FIRST EXPERIMENT

- Lot No. 1.....34 γ/α tocopherol.
 Lot No. 2.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 25 γ of tocopherols.
 Lot No. 3.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 50 γ of tocopherols.
 Lot No. 4.....Non-saponifiable fraction of wheat-germ oil which by chemical titration contains 100 γ of tocopherols.
 Lot No. 5.....68 γ/α tocopherol.
 Lot No. 6.....Controls, no supplement.

SECOND EXPERIMENT

- Lot No. 1.....45 γ/α tocopherol.
 Lot No. 2.....15 γ/α tocopherol.
 Lot No. 3.....Oil of wheat germ which by chemical titration contains 30 γ of tocopherols.
 Lot No. 4.....Oil of wheat germ which by chemical titration contains 15 γ of tocopherols.
 Lot No. 5.....Oil of wheat germ which by chemical titration contains 60 γ of tocopherols.
 Lot No. 6.....Controls, no supplements.

The solutions of alpha-tocopherol, oil of wheat germ, and non-saponifiable fraction were prepared every fifteen days, and during this time they were stored at 0°C. From the results (TABLES 2 and 3), the following facts can be noted: the tocopherol-fed controls behaved in the same way as the controls of the previous investigation (1940), since the animals receiving 34, 45, and 68 gammas of alpha-tocopherol per day did not show any histological lesion or any decrease in weight of the testicle. On the other hand, those which received 15 gammas showed histological lesions in 66 per cent of the animals. These lesions were accompanied by a decrease in the weight of the testicle. Under our experimental conditions, when animals of more than 300 grams give a fresh testicle weight of 1.2 grams, the existence of histological lesions equal or superior to grade 2 in the Mason scale may be confirmed.

The animals which received the non-saponifiable fraction of wheat-germ oil, reacted in such a way as to indicate that the biological activity of this material is approximately a third of what the chemical determination of the tocopherols would imply. The oil of wheat germ, on the contrary, has proved to have a biological activity which is approximately half of what its chemical analysis indicates. These figures should not be taken as exact but as approximate, since the elaboration of this method is still in its early stage. Nevertheless, these figures suggest that by using the prevention of testicular atrophy as quantitative criterion it is possible to gain an approximate idea of the biological activity of a product with respect to its content of vitamin E.

To determine the possibility of obtaining protection against testicular atrophy paralleling the amount of vitamin E received, when only one dose of vitamin E was administered, which would avoid the difficulties of the afore-mentioned method requiring the daily administration of supplements, a third experiment was made. For this, six lots of eight animals were used. They were given only one supplement of alpha-tocopherol when they were

TABLE 2
FIRST ASSAY

Lot. no.	Daily supplement	Final weight average	Testis weight (average)		Results: histological examination (Mason scale)					
			fresh	dried						
1	34 γ tocopherol	333	1.44	0.20	N	N	N	N	N	N
2	Nonsaponifiable fraction of wheat-germ oil which, measured by the Emmerie-Engel method, contains 25 tocopherol	332	0.72	0.09	4	4	4	3	5	5
3	Ditto 50 γ tocopherol	339	0.83	0.11	N	3	5	5	5	5
4	Ditto 100 γ tocopherol	349	1.50	0.21	N	N	N	N	N	N
5	68 γ tocopherol	333	1.48	0.21	N	N	N	N	N	N
6	none	315	0.75	0.09	5	5	5	5	5	5

TABLE 3
SECOND ASSAY

Lot no.	Daily supplement	Final weight average	Testis weight (average)		Results: histological examination (Mason scale)					
			fresh	dried						
1	45 tocopherol	353	1.42	0.20	N	N	N	N	N	N
2	15 tocopherol	337	1.33	0.18	N	2	4	N	2	1
3	Wheat germ oil which measured by the Emmerie-Engel method contains 30 tocopherol	345	0.82	0.10	5	4	4	4	4	3
4	Ditto 15	326	0.68	0.09	5	5	5	5	4	4
5	Ditto 60	343	1.49	0.21	N	N	N	N	N	N
6	none	333	0.84	0.09	5	5	5	5	5	5

TABLE 4
THIRD ASSAY

Lot no.	Unique supplement	Final weight	Testis weight (average)		Results: histological examination (Mason scale)							
			fresh	dried								
1	6 mg. tocopherol	287	1.48	0.21	N	N	N	N	N	N	N	N
2	4 mg. tocopherol	271	1.57	0.22	N	N	N	N	N	N	N	N
3	3 mg. tocopherol	300	1.42	0.20	N	N	N	N	N	N	N	2
4	2 mg. tocopherol	293	1.20	0.15	5	N	5	3	N	N	5	5
5	1 mg. tocopherol	293	1.17	0.14	5	N	N	3	4	2	5	5
6	None	272	0.74	0.10	5	5	5	5	5	5	5	5

40 days old. This is the period at which, according to Mason, the animals show a critical requirement of vitamin E. At that period, the lack of this vitamin produces an irreversible change in the germinative epithelium which always leads to testicular degeneration.

Six lots of animals were used in this investigation (TABLE 4). The animals which received either 6 or 4 mg. of α -tocopherol were protected in 100 per cent of the cases; those receiving 3 mg. were protected in 87 per cent; those receiving 2 in 37 per cent; and those receiving 1 in 25 per cent. The animals which did not receive any supplement showed testicular atrophy of "grade 5." These results indicate that a certain parallelism exist between the dose of vitamin E received and the protection against testicular lesion.

Comments

A series of investigations has been undertaken to determine the possibility of using, as a biological method of vitamin E assay, the prevention of testicular atrophy. The first experiment indicates that litter mates vary no more than 10 days in the time of onset of testicular atrophy when their diet lacks vitamin E. Therefore, it may reasonably be assumed that, if animals suffer complete testicular atrophy within the last ten days of an assay, while the litter mates do not present any testicular lesion, it is because the latter have been protected by the supplement given to them. On this supposition, three investigations were made. In the first and second, vitamin E was given daily from the 30th day of the assay period to the end of the experiment; in the third one, only one supplement was administered. The results obtained indicate that, as was demonstrated by Mason and by us in 1940, the daily administration of alpha-tocopherol in a dose of about 35 gammas prevents testicular lesions in 100 per cent of the animals, and in a dose of only 15 gammas in 35 per cent. The administration of wheat-germ oil and of the non-insaponifiable fraction of wheat-germ oil prevents testicular atrophy to a degree indicating that its biological activity is between a half and a third of what the chemical determination (effected according to the Emmerie-Engel method) would show. This fact is easily explained, as the alpha-, beta-, and gamma-tocopherols are determined together by this chemical method and the existence of different activities for these three tocopherols is well known. The administration of one dose of alpha-tocopherol protected the animals against testicular atrophy. The percentage seemed to be in proportion to the amount of vitamin E received. The method thus established is difficult and long, but it is possible that, by standardizing the experimental conditions to obtain animals with a minimum of vitamin E reserves, such as has been done by Mason in the biological method used at present, the period of dosage of this vitamin may be reduced to approximately one month. According to Mason and Bryan, the period during which animals stay protected by the reserves accumulated during lactation is estimated as 47 days. In this case it would be possible to establish a simple and practical method for the biological determination of vitamin E.

Summary

A method for the biological determination of vitamin E, using as quantitative criterion the prevention of testicular atrophy of the male rat, is proposed. This method, under the conditions established at present, is long, but it seems possible to establish a simple and quick method, once the experimental conditions are standardized.

Discussion of the Paper

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): While bio-assay techniques are currently being supplanted by chemical methods, they will always remain a necessity as a specific check on chemical procedures. We have long recognized the striking sensitivity of the male rat to small amounts of tocopherol, as reflected in the delayed onset of testis damage. It is gratifying to see the development of a bio-assay method utilizing this phenomenon. While the procedure may take a longer experimental period than that involving the female rat, it may compensate in part by utilizing the customary surplus of males in a rat colony.

VITAMIN E STUDIES ON MICE WITH SPECIAL REFERENCE TO THE DISTRIBUTION AND METABOLISM OF LIPIDS

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Introduction

In vitamin E studies, most experiments have been performed on rats, with comparatively few on mice. In order to find manifestations of deficiency common to all species and a common denominator for its basic functions, it seems to be of interest to evaluate species variations in nutritional deficiencies and requirements. Vitamin E is still a controversial issue in respect to its influence on the human organism and in respect to its fundamental physiological or biochemical significance.

With the view of finding some data which could act as a contribution to our knowledge on vitamin E, experiments were initiated in 1941 and carried on until 1946 in Edinburgh. At present, part of the collected material is gradually being examined and analyzed in Ottawa. This paper intends to give a general survey of results so far obtained.

Material and Methods

Over 250 mice (British M.R.C. strain) have been used for experiments from a colony reared under uniform conditions throughout the five years of experimentation.

The vitamin E deficiency was brought into effect by feeding a slightly modified diet No. 427 of Emerson and Evans,¹ containing 22 per cent of lard and 2 per cent of cod-liver oil. The vitamin E-deficient mice were compared with their litter-mate sisters and brothers kept on the same diet supplemented with 2.5 mg. daily (six days a week) of synthetic dl-alpha-tocopheryl acetate, kindly supplied by Roche in England.† The experimental mice were also compared with their siblings kept on a standard laboratory diet, the 14 per cent dried-milk rat-cake of the North-Eastern Agricultural Co-operative Society of Aberdeen in Scotland. More details on general experimental procedure can be found in a previous paper.² The experiments have been planned primarily to show the effect of prolonged, long-term, deficiency.

Results

The influence of vitamin E deficiency or of the fairly rich (2.5 mg. daily) supplement on mice can usually be noted in many organs. No marked changes, however, were encountered in animals on experiment for one year or less. A mouse can stand the vitamin E deficiency for a rather long period.

* With technical assistance of J. Olszowski.

† We owe thanks to Roche Products Limited, Welwyn Garden City, Herts, England for granting us this generous supply.

One female managed to live for 791 days on the minus E diet and actually died before our eyes while being examined by two of us (Z. M. and T. R.). For the sake of illustration, we include a few interesting points from her protocol (TABLE 1). The first sign of deficiency in this animal

TABLE 1
EXTRACT FROM THE PROTOCOL OF THE ANIMAL ♀ B₂₆.

Group 3.

Experiment VIII.

Parents: ♀ F₁₈ x ♂ S₂₃.

Animal: ♀ B₂₆.

Born: January 2, 1942.

Weaned: January 23, 1942.

E-deficient 427 diet started: January 23, 1942.

May 26, 1943. Weight 48.8 g.

June 2, 1943. Weight 48.5 g. looking well.

July 7, 1943. (After 530 days of experimental feeding). Placing hind limbs slightly apart. Flattening of the rump.

July 20, 1943. Weight 32.4 g. Minimal bristling of the coat.

Dec. 21, 1943. Weight 25.0 g. Lean. The rump flat. Hind limbs paralyzed. The abdomen trails on the ground. Hair stands on end.

January 15, 1944. Hind limbs in rigid extension. Grasping-like rigid contractions on mechanical stimulation of paws.

Feb. 17, 1944. Cannot lift the trunk from the ground. Only right hind limb works, left completely paralyzed.

March 24, 1944. Weight 22.7 g. Complete paralysis of both hind limbs. Paresis of front limbs. Bald patches in the fur on the dorsum. Tail-end necrotic. Right cornea opaque.

During actual examination suddenly dies (this is the 791st day of E-deficient diet).

Autopsy: No adipose tissue, but interscapular fat present. Intestines of yellowish brown color. Uterus very thin and discolored. Ovaries large and yellow. Suprarenal glands large and light. Marked lordosis of vertebral column in lower cervical and upper thoracic regions.

was a slight drop in weight after 495 days of minus E feeding, but no other changes were found on physical examination. A little later, very slight locomotor disturbance was noted: placing apart of the hind limbs. As time went on, extreme leanness developed and locomotor disturbance became aggravated. Such changes as bristling of hair, bald patches on the fur coat, terminal necrosis of the tail, slight opacity of the cornea, and lordosis of the vertebral column, noted in this particular animal, do not belong to the regular findings of our E-deficient mice. On the other hand, the leanness, *i.e.*, lack of adipose tissue, and the brown discoloration of organs should be considered as unfailing, typical features in the picture of our minus E mice, and both these characteristics may be regarded as being more or less connected with the distribution and metabolism of lipids in the organism. The locomotor disturbance and the changes in the genital organs seem to belong to additional, subsidiary, manifestations of deficiency in mice. They could be regarded as "by-products" of the deficiency; the main "product" probably is a disturbance in metabolic arrangement of lipids.

The results, in general, may be surveyed under the following headings: (a) locomotor disturbance; (b) changes in the genital organs; and (c) disturbance in the distribution of lipids.

(a) *Locomotor disturbance.* The muscular dystrophy in mice was noted by Pappenheimer³ in 10 per cent of animals aged between 36 and 439 days. The incidence of dystrophic symptoms and signs in our minus E mice was very high (TABLE 2), starting with the group of animals between one and a quarter to one and a half years of age.

The onset of the first clinical signs of locomotor disturbance (TABLE 3), falls on the 403rd day of the E deficient feeding on the average. The onset is not sudden, but very insidious and inconspicuous. The changes were usually very slowly aggravated, so that the longest survival with untreated lesions from onset to death was 262 days. The locomotor disturbance was clinically manifested by paresis, paralysis, and rigid extensions and flexions of the limbs, especially in the hind limbs (FIGURE 1).

Preliminary histologic examination of muscles in a few cases showed changes ranging from one-plus to three-plus according to Madsen, McCay, and Maynard's⁴ classification, but the results of the preliminary examination of the nervous system have so far been negative.

(b) *Changes in the genital organs.* Serial sections were made of testes and ovaries. The testes of mice are very resistant to the E deficiency, as Bryan and Mason⁵ and Goettsch⁶ have shown in experiments carried on for 400 days, and Pappenheimer³ in experiments of 439 days of minus E feeding.

Under the experimental conditions of our E-deficient mice, degenerative changes became evident after about one year and a half on deficient diet (FIGURES 2 a-d), which cannot be attributed to the influence of old age only, as the control litter-mate brothers showed no such changes. In the E deficient males, the testes showed brown discoloration and the internal genital organs were generally diminished in size (FIGURES 2 e, f). (Details will be published soon elsewhere.)

Detailed serial examination of the ovaries was performed and published by Menschik.² From the results it can be concluded that E-deficient mice show a suppression in the amount of all the elements originating from germinal epithelium; that is, the amount of follicular cells, primordial ova, and interfollicular tissue. The animals on diet supplemented with vitamin E show, on the contrary, an increased amount of these elements. It can be added that the female kept longest on plus E diet (supplemented through 633 days with 2.5 mg. daily of tocopherol) developed a unilateral granulosa-cell ovarian tumor. This observation is reported, but no conclusion should be drawn from a single case.

TABLE 2 (See opposite page).

Note. In all these recorded experiments the vitamin E-deficient diet was started on the day of weaning: between 21st and 24th day of age. As the experiments proceeded the animals were killed at intervals (some died).

Total number of E-deficient animals under observation in the analysed experiments: ♀ 45, ♂ 27, total 72. Total number of E-deficient animals which showed locomotor disturbance: ♀ 24, ♂ 13, total 37.

Conclusion. The greatest incidence of dystrophy is in age group between 457-548th day, when, of 16 females alive, 14 showed locomotor signs, and, of 7 males alive, 6 showed changes.

TABLE 2 (For description see facing page)
THE INCIDENCE OF LOCOMOTOR DISTURBANCE IN RELATION TO THE AGE

EXPERIMENT	NUMBER OF ANIMALS ON VITAMIN E DEFICIENT DIET BY AGE GROUPS															
	NO.	At the START	0-183 DAYS 0-1/2 YEAR		184-274 DAYS 1/2 - 3/4 YEAR		275-365 DAYS 3/4 - 1 YEAR		366-456 DAYS 1 - 1 1/4 YEAR		457-548 DAYS 1 1/4 - 1 1/2 YEAR		549-639 DAYS 1 1/2 - 1 3/4 YEAR		640-730 DAYS OR 1 3/4 - 2 YEARS OR MORE	
			ALIVE	DYSTROPH.	ALIVE	DYSTROPH.	ALIVE	DYSTROPH.	ALIVE	DYSTROPH.	ALIVE	DYSTROPH.	ALIVE	DYSTROPH.	ALIVE	DYSTROPH.
		♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
II JULY 4, 41		6	—	—	—	—	—	1	—	—	—	—	—	—	—	—
VI DEC. 11, 41		8	8	—	—	—	—	2	3	—	2	—	—	—	—	—
VII JAN. 2, 42		7	—	—	—	1	—	7	—	1	—	6	—	1	—	—
IX JAN. 11, 42		2	5	—	—	—	—	—	2	—	2	—	—	—	—	—
X MAR. 14, 42		8	6	—	—	4	—	7	6	1	1	5	1	—	—	—
XI APR. 8, 42		4	1	—	—	—	—	3	1	2	1	—	—	—	—	—
XII APR. 25, 42		3	5	—	—	—	—	3	3	1	2	2	1	—	—	—
XIII MAY 11, 42		3	1	—	—	1	—	2	1	2	—	—	—	—	—	—
XIV MAY 19, 42		4	—	—	—	—	—	4	—	2	—	1	—	—	—	—
TOTAL NUMBER OF —E ANIMALS		45	27	36	24	31	20	29	15	25	10	16	7	5	2	1
TOTAL NUMBER OF DYSTROPHIC ANIMALS PER GROUP.					—	1	5	5	3	9	3	14	6	4	2	1



FIGURE 1 (For description see facing page)

TABLE 3
THE TIME OF ONSET OF FIRST SIGNS OF LOCOMOTOR DISTURBANCE

Duration of diet	Number of animals		
	♀	♂	Total
Under ½ year (0–183 days)	0	0	0
½–¾ year (184–274 days)	1	5	6
¾–1 year (275–365 days)	6	2	8
1–1¼ year (366–456 days)	6	2	8
1¼–1½ year (457–548 days)	10	3	13
1½–1¾ (549–639 days)	0	0	0
1¾–2 years or more (more than 640 days)	1	1	2
Total number of dystrophic animals	24	13	37

The earliest onset: after 209 days of E-deficient feeding.
The latest onset: after 648 days of E-deficient feeding.
The longest survival with dystrophy from onset to death: 262 days.
The average onset falls on the 403rd day of E-deficient feeding.

The uteri of vitamin E-deficient mice show discoloration and reduction in size, in comparison with controls and with E rich animals (FIGURE 2 g–i).

(c) *Disturbance in the distribution of lipids.* The most striking and common feature in mice on E deficiency was the complete lack of subcutaneous and subperitoneal adipose tissue, except for the interscapular fat, the so-called hibernating glands.⁷ The amount of the brown adipose tissue was increased and was of a deeper brown color than in controls.

When the diet was supplemented with tocopherol, the animals showed conspicuous obesity⁸ in all possible depots, including the perimysial connective tissue. In these animals the interscapular fat gave a spurious appearance of being enlarged, but in reality it was replaced in large part by common adipose tissue, the usual white fat. Studies on the correlation between the brown fat and vitamin E in mice are not yet concluded. Besides the general obesity, fatty changes appear in the parenchymal cells of some organs, for instance, adrenals (FIGURE 3, *f*) and liver (FIGURE 4, *f*), in animals in which the fat-rich E-deficient diet was supplemented with tocopherol. Histochemical studies, carried out especially in the liver,⁹ showed fatty metamorphosis in plus E animals, accompanied sometimes with consecutive formations of watery vacuoles (FIGURE 4, *v*). The fatty changes in the liver of E-rich mice present chiefly neutral fat, giving pink red reaction with Nile blue sulphate.

It should be mentioned that minute granulès of a brown non-lipoid pigment, the nature of which has still to be investigated, was found incon-

FIGURE 1 (See opposite page). Photographs of mice with locomotor disturbance:
(a) Male, B-49, photographed after 604 days of vitamin E deficiency, and 183 days after the onset of noticeable locomotor disturbance.
(b) The same male (B-49), after 683 days of E-deficient diet, after 262 days of locomotor disturbance. This animal was killed soon after this photograph was taken.
(c) Female, B-69, photographed after 582 days of E deficiency: 215 days from the onset of dystrophy.
(d) The same female (B-69) after 587 days of minus E diet, and after 220 days from the onset of first signs of locomotor disturbance. Owing to its very poor condition, this animal was killed soon after this photograph was taken.
(e) Female, B-26, photographed after 758 days of vitamin E deficiency, 228 days from the onset of locomotor disturbance.
(f) The same female (B-26) after 786 days of E-deficient feeding, and after 256 days of locomotor disturbance. This animal died suddenly during examination 5 days after this photograph was taken.

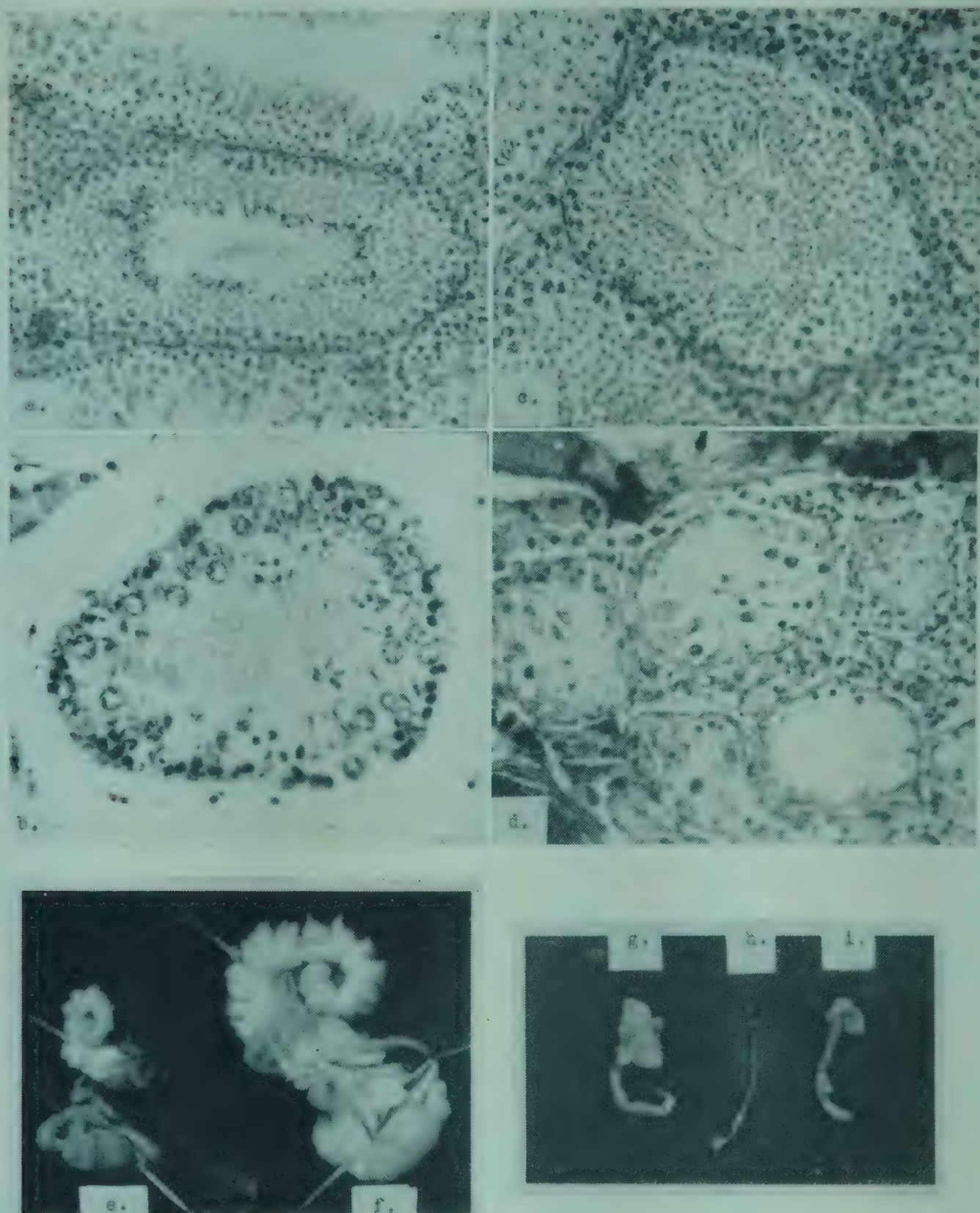


FIGURE 2.

FIGURES 2a.-d. Photomicrographs of testicular sections

- (a) A mouse on a control, standard laboratory diet, age 555 days. Mayer's hemalum and eosin. $\times 210$;
- (b) A litter-mate brother of the preceding (a) animal, age 555 days, kept for 531 days on vitamin E-deficient diet. Mayer's hemalum and eosin. $\times 380$;
- (c) A mouse from a control, standard laboratory diet, age 834 days. Mayer's hemalum and eosin. $\times 250$;
- (d) An animal at the age of 706 days, after 685 days of vitamin E deficiency. Mayer's hemalum and eosin. $\times 250$.

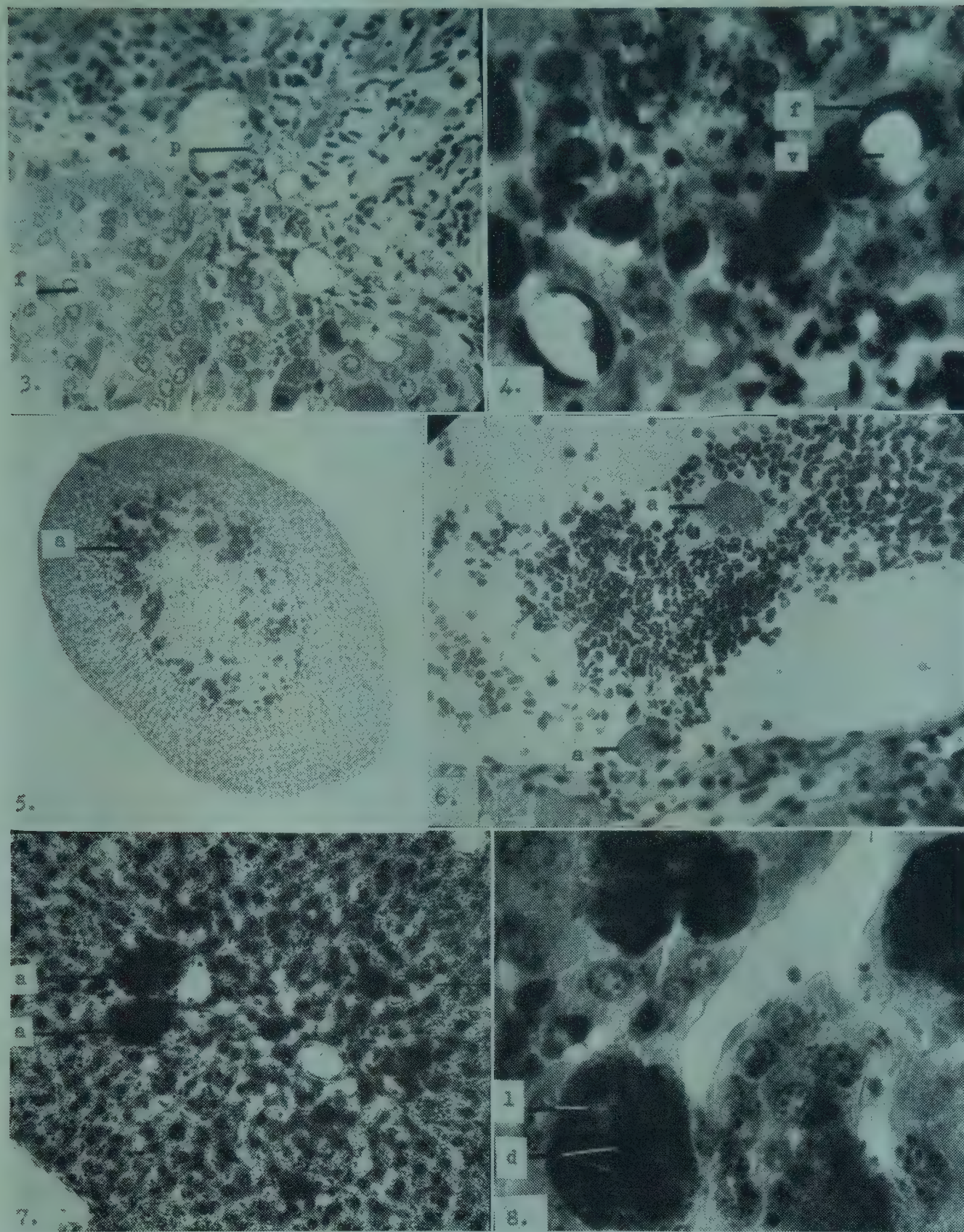
FIGURES 2e. and f. Photographs of the internal genital organs of two male mice:

- (e) A male which survived longest on vitamin E-deficient diet, age 706 days, kept for 685 days on the diet;
- (f) The oldest control male kept on the standard laboratory diet during its whole life, age 834 days.

FIGURES 2g.-i. Photographs of ovaries and uteri of three litter-mate sisters, killed at the age of 611 days:

- (g) A mouse kept for 587 days on E-deficient diet supplemented with tocopherol;
- (h) An E-deficient mouse after 587 days of the deficiency;
- (i) A control mouse, fed with the standard laboratory diet.

stantly in the phagocytes of the liver, of adrenals (FIGURE 3, *p*), and of the subcutaneous and subperitoneal adipose tissue. This pigment was encountered in animals on fat-rich E-deficient diet, regardless of whether the diet was supplemented with tocopherol. It was never noted in control animals on the standard laboratory diet.



FIGURES 3-8.

FIGURE 3. Photomicrograph of suprarenal section. A female mouse, age 392 days, kept for 369 days on E-deficient diet supplemented with 2.5 mg. of tocopherol daily during the entire period of diet. Mayer's hemalum and eosin. $\times 360$. f—fatty metamorphosis of parenchymal cells in zone X; p—minute granules of non-lipoid brown pigment.

FIGURE 4. Photomicrograph of liver section. A female, age 486 days, kept on a plus E diet (fat-rich, E-deficient, plus 2.5 mg. of tocopherol daily) for 465 days. Frozen section. Mayer's hemalum and Sudan III. $\times 700$. f—fatty metamorphosis; v—watery vacuoles.

FIGURE 5. Photomicrograph of suprarenal section. A male mouse, age 552 days, kept for 530 days on vitamin E-deficient diet. Mayer's hemalum and Sudan III. $\times 35$. a—acid-fat pigmented substances.

FIGURE 6. Photomicrograph of suprarenal section. A female mouse, age 770 days, after 748 days of vitamin E deficiency. Mayer's hemalum and eosin. $\times 360$. a—acid-fast pigmented substances.

FIGURE 7. Photomicrograph of liver section. Female mouse, age 619 days, kept for 598 days on E deficient diet. Frozen section. Mayer's hemalum and Sudan III. $\times 140$. a—acid-fast material.

FIGURE 8. Photomicrograph of a liver section. The same animal as in the preceding (fig. 7) photomicrograph. Frozen section. Sudan III staining in Romeis's¹² modification. $\times 700$. d—darker phase (granules) of acid-fast material; l—lighter phase (globule) of this material.

Besides the leanness, the appearance in many organs (gonads, liver, spleen, lymphnodes, adrenals, and in scattered phagocytes of many tissues¹⁰) of pigmented acid-fast material was the second most common characteristic

in the vitamin E-deficient animals. This material presents a widespread distribution. In adrenals, for instance, it not only accumulates in adjacent parts of the cortex and the medulla, where it is found rather early (FIGURE 5, *a*), but it can be found in all parts of the gland, sometimes even in the lumen of a vessel (FIGURE 6, *a*).

The liver can serve as another example. There, single cells or groups of liver cells were loaded with these pigmented substances, which were insoluble in fat solvents, very slightly stainable with eosin, and well stainable with light green and orange G (probably signaling proteins chiefly of pH range between 11 and 13). These substances were acid-fast and gave a blue reaction with Nile blue sulphate, indicating the presence of unsaturated fatty acids. They also gave a plus result when tested with Smith-Dietrich's method for phospholipids and a positive Schulze's reaction¹¹ for cholesterol. Furthermore, it was shown that they were also sudanophilic (FIGURE 7).

These substances seem to appear in two phases: one, more concentrated, darker, and in the form of rather small granules (FIGURE 8, *d*) located inside another second phase, which is in the form of a larger and lighter globules, (FIGURE 8, *l*), presenting, however, the same histologic and histochemical reactions as the darker phase.

In general, these pigmented acid-fast substances show lipo-proteic characters.

Discussion and Conclusions

On the basis of the observations just presented, it may be said that, in mice, the changes of the lipid distribution are in the foreground when influence of vitamin E is considered. Plus E animals show a general increase in the amount of neutral fat, while in minus E mice this neutral fat disappears, with a simultaneous appearance of lipo-proteic substances, although the animals received exactly the same—except for vitamin E—fat-rich diet. The amounts of neutral fat and of lipo-proteic material are inversely proportional. Moreover, this relationship is a function of vitamin E supply. On such grounds, it may be suggested that vitamin E influences lipid metabolism in mice in such a way that the ingested and/or the tissue lipids are converted mainly into neutral fat in the presence of an adequate or rich supply of tocopherol. When the deficiency of this vitamin occurs, the fat administered or the tissue fat, or both undergo abnormal or abortive metabolic changes, resulting in the absence of histochemically detectable neutral fat and in the formation of pigmented acid-fast substances, composed of unsaturated fatty acids, phospholipids, cholesterol, and proteins.

Summary

Long-term experiments, on more than 250 mice, have shown that muscular dystrophy and testicular degeneration are encountered in some instances. Changes in distribution of lipids are found in all animals.

Mice in which the fat-rich E-deficient diet was supplemented for over a year with 2.5 mg. daily of synthetic tocopherol have shown an increased

amount of adipose tissue (except for the brown fat) and fatty changes in some organs.

In vitamin E-deficient mice, with the disappearance of neutral fat and the development of extreme leanness, there developed acid-fast pigmented substances in many locations of the body. In the composition of these substances, unsaturated fatty acids, phospholipids, cholesterol, and proteins take part.

These observers suggest an influence of vitamin E in enzymatic reactions during lipid metabolism.*

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Discussion of the Paper

DR. K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): The testicular degeneration observed in the vitamin E-deficient mouse by Dr. Menschik, but only after very prolonged feeding approximating two years, is of interest in that both Pappenheimer and I failed to note any injury after a year or more of feeding. The histologic changes just described might be related to advanced senility. Further data will be needed to settle this question. The fact that the changes are unlike those seen in the E-deficient rat does not mean that they cannot be specifically related to vitamin E deficiency. I have thought for a long time that testicular damage in other species should resemble that in the rat, especially in the irreversibility of the degenerative changes. I should like to place on record the fact that this is not necessarily the case. In studying the phenomenon in the vitamin E-deficient hamster, I have observed a progressive and extensive degeneration of the testis which can be quite adequately repaired by vitamin E therapy.

* Menschik is now starting an investigation of vitamin E influence on embryonic and fetal development, with a histochemical approach and with special attention to lipids. The work is being done at the University of Ottawa, School of Medicine, Ottawa, Canada, with the aid of a grant from the National Research Council, Ottawa, Canada.

TEN-YEAR INCIDENCE OF FIELD ENCEPHALOMALACIA IN CHICKS AND OBSERVATIONS ON ITS PATHOLOGY

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Under experimental conditions, a high-fat vitamin E-deficient diet is known to induce in chicks nutritional encephalomalacia,¹ in ducklings muscular dystrophy,² and in poult myopathy of the gizzard,³ whereas a similar low-fat diet produces in chicks exudative diathesis.⁴

While corresponding pathologic entities have been observed under field conditions, only encephalomalacia is of relatively common occurrence.⁵ Exudative diathesis is seen occasionally in chicks and poults, particularly in conjunction with high salt diets or prolonged exposures to coal-tar fumes. In addition, two clinically inapparent conditions should be mentioned on account of their pathologic similarities, namely: muscular dystrophy in ring-necked pheasants,⁶ resembling the experimental disease in ducklings; and myopathy of the gizzard in chickens,⁷ resembling that in poults.

There are relatively few accounts in the literature of the spontaneous incidence of encephalomalacia in chicks and its associated pathology. The present communication is concerned with statistical and pathologic observations on E-avitaminosis in birds, based upon the State diagnostic records of the past 10 years. As a rule, two specimens from each consignment or lot were sacrificed for subsequent histopathologic and neuropathologic examinations by routine paraffin technics.

Incidence of Field Encephalomalacia

In using diagnostic records as a measure of the incidence of any disease, it is realized that the data do not represent vital statistics, which are unavailable. It is known that diagnostic figures are influenced by the distance of the flocks from the laboratory and the economic fluctuations of the poultry population. Nevertheless, it is easily seen that, percentagewise, the yearly fluctuations in the incidence are much greater than those of the poultry population as a whole. Furthermore, the principal manifestations of field encephalomalacia are well known to the experienced poultry man under the name of "crazy chick" disease and occur during the brooder stage, when the poultry man is particularly on the alert for clinical abnormalities. Thus, there is little doubt that cumulative laboratory diagnostic records reflect, by and large, the relative incidence and economic importance of a given poultry malady in the field.

Since the first recognition of field encephalomalacia on a histologic basis,⁵ the incidence has been recorded for the years 1936 to 1938 as amounting to 31, 15, and 13 case lots, respectively.⁸ During the period 1939 to 1948, the available data indicate an incidence of 16, 32, 25, 23, 8, 15, 3, 23, 11, and 62 known cases, respectively. Thus, during the first 12 years, the average incidence ranged from a high of 31 in 1936 and 32 in 1940 to a low

of 3 in 1945, with an average of 18 per year, while the incidence in 1948 was almost three and one-half times the yearly average.

The reasons for the yearly fluctuations in the incidence of field encephalomalacia are unknown. In the past, speculative attempts have been made to correlate such variations with the quality of the available corn crop, especially with so-called "heated" corn. There is some experimental evidence that fermentable substances in the mash increase the incidence.⁹ It is interesting to note that the late war years, in spite of their attendant difficulties in the compounding of poultry feeds, brought about a significant reduction in the incidence. In 1947, a new type of broiler ration, characterized by high energy and low fiber values,¹⁰ was first fed commercially in Connecticut without any unusual increase of encephalomalacia cases. The same type of ration was again used widely in 1948 with excellent results, save for the high incidence already recorded. As the original formula of the feed called for 19 per cent animal protein supplements, the demand may have been satisfied by ingredients of inferior quality, especially with respect to high fat content. Later modifications of the formula, with a low percentage of liver meal, seemed to bring about a decrease in the incidence. Apparently the same effect was obtained by one feed mixer from the addition of a relatively small amount of wheat-germ oil.

Analysis of the data for 1948 showed an affected chick population of 235,515, of standard breeds, ranging in age from 1 to 10, average 5, weeks. The reported mortality varied from zero, except for the laboratory specimens, to 34.7, average 3.6 per cent. Instances of unusually high mortality were probably due to complicating factors such as coccidiosis and Newcastle disease. The monthly incidence for May to October was 2, 25, 16, 9, 6, and 4 lots, respectively, and thus showed a seasonal peak only one month later than that ascertained for 1936 to 1938.⁸

The above data include cases of acute field encephalomalacia verified in the laboratory, but not those characterized as chronic lesions or the many cases reported by word of mouth. Feeding experiments with suspected samples obtained in the field produced a few affected chicks, comparatively insignificant in number.¹¹ Naturally, speculation is rife as to the probable incidence in 1949.

Pathologic Observations

A detailed description of the neuropathology of nutritional encephalomalacia was furnished by Wolf and Pappenheimer.¹² The authors pointed out that the acute lesions are primarily indicative of ischemic necrosis (FIGURES 1, 6, 14) but that it is not uncommon to find other areas of the brain in various stages of repair.

Localization. The preferential localization of the acute lesions in the cerebellum has given rise to the assumption that the majority of cases of field encephalomalacia can be diagnosed on gross examination of the brain. While this may hold true on a lot basis, the present work has brought out the fact that individual specimens often show encephalomalacic foci exclusively in the corpus striatum or in the medulla (FIGURE 2) where they

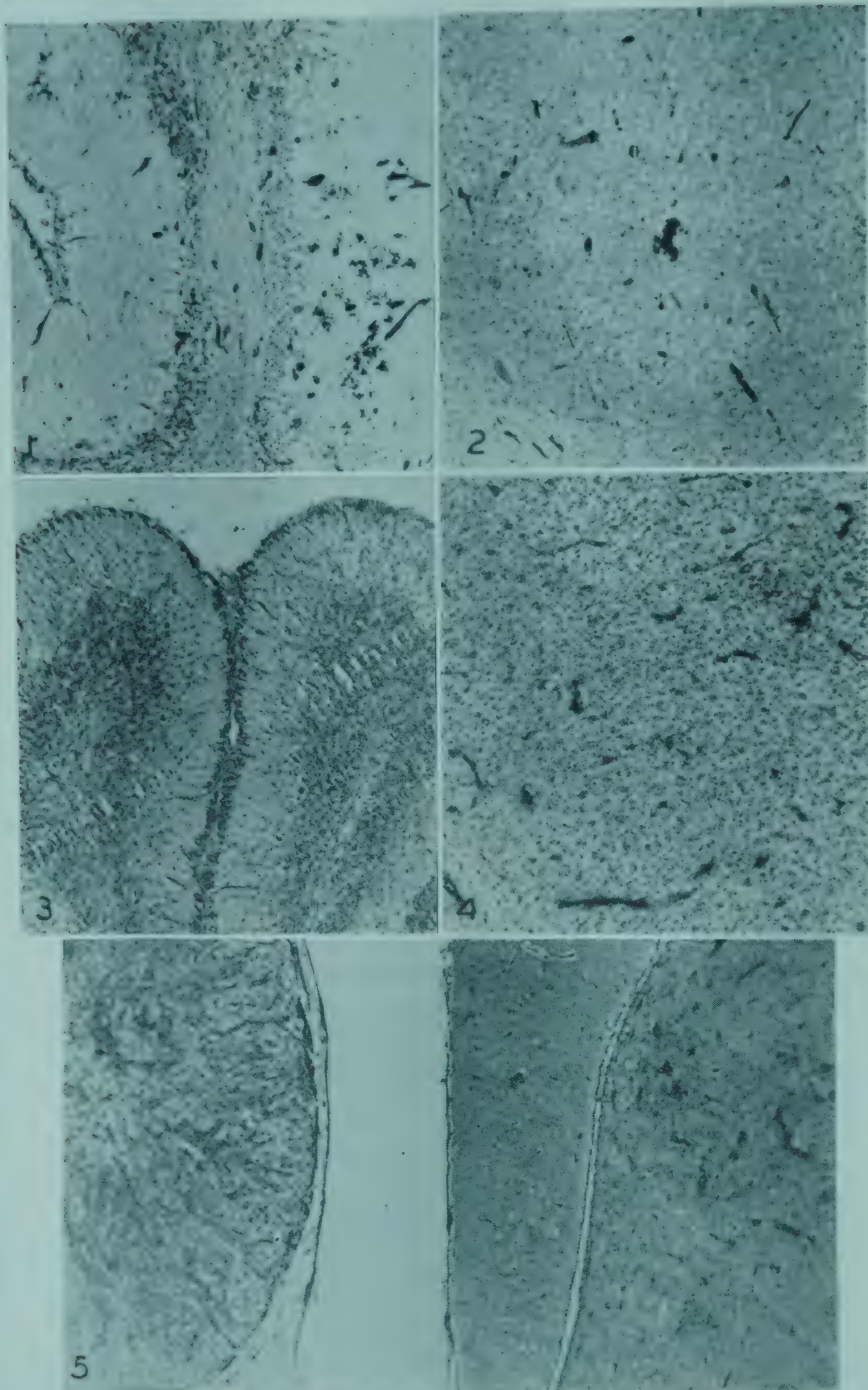


FIGURE 1-5 (For description see facing page)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-triosin, except when stated otherwise. 80 X. (Slightly reduced).

would be overlooked on gross examination even if of macroscopic size. Smallness of the brain in very young chicks likewise mitigates against gross recognition.

Fibrosis of Cerebellum. Of particular interest was the relatively frequent occurrence of cerebellar fibrosis not associated with ischemic necrosis. Chicks so affected showed indefinite symptoms of incoordination, paresis, and tremor which could not be differentiated clinically from those caused by avian encephalomyelitis (FIGURE 14) or Newcastle disease.

The lesion was seen occasionally in one-week-old specimens (FIGURE 3) but was otherwise encountered in chickens of various ages (FIGURES 5, 7, 9, 10) up to 32 weeks. It has been observed in association with the specific neuropathologic manifestations of Newcastle disease, avian encephalomyelitis, and neural lymphomatosis and thereby may give rise to confusion.

Incipient cases show the capillaries which extend at right angles from the pia into the cerebellar molecular layer (FIGURE 7), to be thickened and accompanied by slight glial proliferation. In one-week-old chicks, the external granular layer may still be present or in a state of hyperplasia (FIGURE 3). Gradually the glial proliferation is replaced by connective tissue which also becomes prominent in the capillary walls, as revealed by special staining techniques (FIGURE 9). The internal granular layer is likewise permeated by fibrotic tissue and seems to transgress the boundary line of the Purkinje cells, thereby destroying the normal architecture (FIGURE 10). Eventually, the contraction of the connective tissue brings about a scalloped surface of the molecular layer with thick radially arranged vessels.

Cerebellar fibrosis has been seen in individual specimens (FIGURE 7) of lots otherwise showing typical acute lesions (FIGURE 6). Undoubtedly, it represents the counterpart to the experimental lesions described by Wolf and Pappenheimer.¹² Its occurrence at the age of one week suggests parentally transmitted deficiency; its occurrence at later ages, up to 32 weeks, often in association with other affections of the nervous system, suggests that quite a few clinically recovered cases escape routine detection.

Vascular and Adventitial Proliferations. Perhaps even of greater interest than cerebellar fibrosis was the occurrence of large areas of increased vascularity (FIGURES 4, 8), accentuated by various degrees of adventitial cell proliferation and intervascular gliosis (FIGURE 11). Characteristic foci were observed in the medulla, midbrain, thalamus, and particularly in the deeper portions of the corpus striatum; while the hyperstriatal areas remained relatively free (FIGURE 5). The lesions often occurred in association with ischemic or fibrotic lesions (FIGURE 5) in the cerebellum. Although they have been observed in chicks at one week of age (FIGURE 4), they tended to become more pronounced with advancing age (FIGURES 12, 13). Similar lesions have not been observed in uncomplicated cases of

FIGURES 1-5 (See opposite page)

FIGURE 1. 17 days old. Cerebellum. Acute lesion. Pyknosis of granular layers in center and hemorrhages and hyaline thrombosis in right molecular layer.

FIGURE 2. 5 d.o. Medulla. Acute lesion. Triangular malacic focus with apex in low center, hemorrhages and hyaline thrombosis.

FIGURE 3. 7 d.o. Cerebellum. Fibrosis of molecular layer and thickening of external granular layer (in center).

FIGURE 4. 7 d.o. Hypostriatum. Vascular proliferation and intervascular gliosis.

FIGURE 5. 21 d.o. Cerebellum on left shows complete fibrosis. Hyperstriatum accessorium (in center) is normal, hypostriatum to right of lateral ventricle shows marked vascular proliferation.

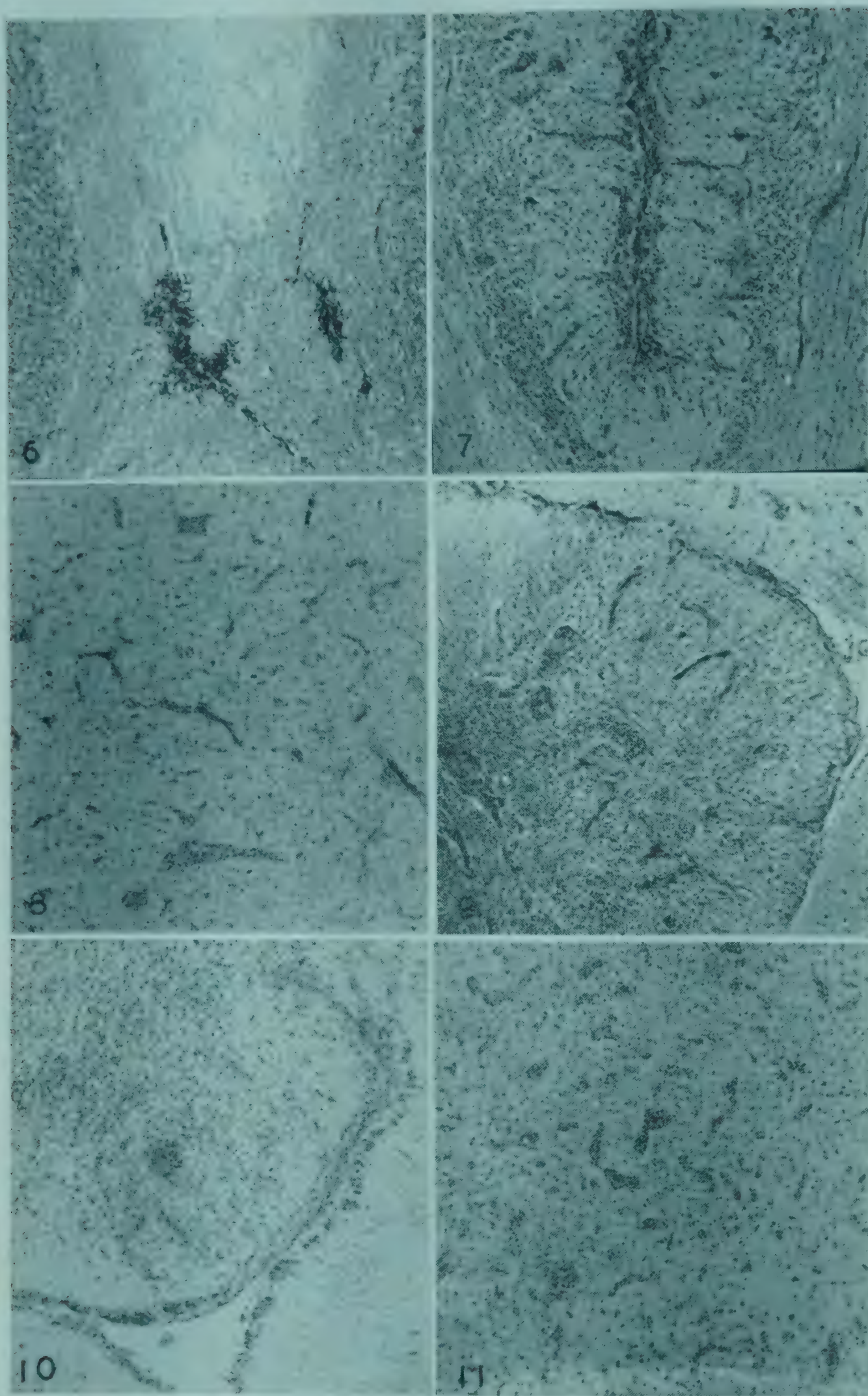


FIGURE 6-11 (*For description see facing page*)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-triosin, except when stated otherwise. 80 X. (Slightly reduced).

other known neuropathologic entities such as avian encephalomyelitis, *etc.*, and are believed to constitute a new morphologic expression of subacute E-avitaminosis in chickens.

Mild, presumably recent cases show large well-delimited foci of proliferated capillaries which are widely spaced and may have a (FIGURES 4, 5, 8) prominent mantle of adventitial cells. Further developed lesions exhibit the same general arrangement, but the vessels are tortuous and thickened (FIGURES 11, 12). Advanced lesions consist of wide conspicuous vessels surrounded by thick bands of adventitial cells (FIGURE 13). The latter elements are somewhat oblong and pale and thereby differ from ordinary perivascular mononuclear cuffs. Without study of the progressive development of these lesions in various age groups, advanced changes would not be suspected as belonging to the pathologic spectrum of E-avitaminosis. Transmission experiments with and attempts to demonstrate toxins in brains so affected have been consistently negative.

Pathologic Relation to A-avitaminosis. Studying the role of vitamin E in chick nutrition, Patrick and Morgan¹³ found this nutrient to be necessary for the utilization of vitamin A in simplified diets. They expressed the opinion that "field encephalomalacia is probably a vitamin A deficiency." To inquire into this relationship, beaks of field encephalomalacia cases were examined by the method of nasal histopathology,¹⁴ which has proved highly sensitive in the detection of subtotal vitamin A deficiencies. In none of the 40 cases examined was there any detectable evidence of A-hypovitaminosis.

In a histologic comparison of the brain lesions in vitamin A- and E-deficient chicks, Adamstone¹⁵ found the former characterized by achromatic pinpoint areas in the brain stem, cerebellum, optic chiasm, and rarely in the cerebrum. Similar unstained focal areas were frequently seen in cases of field encephalomalacia (FIGURE 15), without accompanying evidence of A-hypovitaminosis in the nasal passages. In an experimental study of vitamin A requirements,¹¹ seven lots of day-old chicks received 40 to 1280 I. U. of vitamin A per 100 grams of feed, respectively. Of two chicks sacrificed per lot at 3 weeks of age, all of them, even those receiving the highest doses, showed pinpoint lesions, while only the 40 and 80 U. lots presented specific nasal lesions. At 14 weeks only 2 chicks in the 1280 U. group showed such brain lesions. In a similar experiment with seven lots of poults receiving 150 to 7200 I. U. of vitamin A, respectively, 2 poults per lot sacrificed at 2 and 4 weeks failed to show these brain changes, while 12 of 14 showed them at 6 and 8 weeks, including those birds receiving the highest dosage levels.

Thus, no correlation could be observed between the occurrence of achromatic pinpoint lesions in the brain and specific lesion of A-hypovitaminosis in the nasal mucosa, under either spontaneous or experimental conditions.

FIGURES 6-11 (*See opposite page*)

FIGURE 6. 24 d.o. Cerebellum. Mild acute lesion. Central white matter shows hemorrhages in low center and edema in high center.

FIGURE 7. 24 d.o. Same lot as FIGURE 6. Cerebellum shows marked fibrosis of molecular layer and hyperplastic thickening of external granular layer (in center).

FIGURE 8. 24 d.o. Same specimen as FIGURE 7. Hypostriatum shows proliferation of vessels and early proliferation of adventitial cells (in low center).

FIGURE 9. 28 d.o. Cerebellum. Advanced fibrosis in right upper and lower quadrant of molecular layer. Top left area normal. Masson's trichrome.

FIGURE 10. 28 d.o. Cerebellum shows extensive fibrosis and nearly complete loss of architecture. External granular layer forms a thickened border.

FIGURE 11. 28 d.o. Same specimen as FIGURE 10. Hypostriatum shows marked vascular and adventitial proliferation and intervascular gliosis.

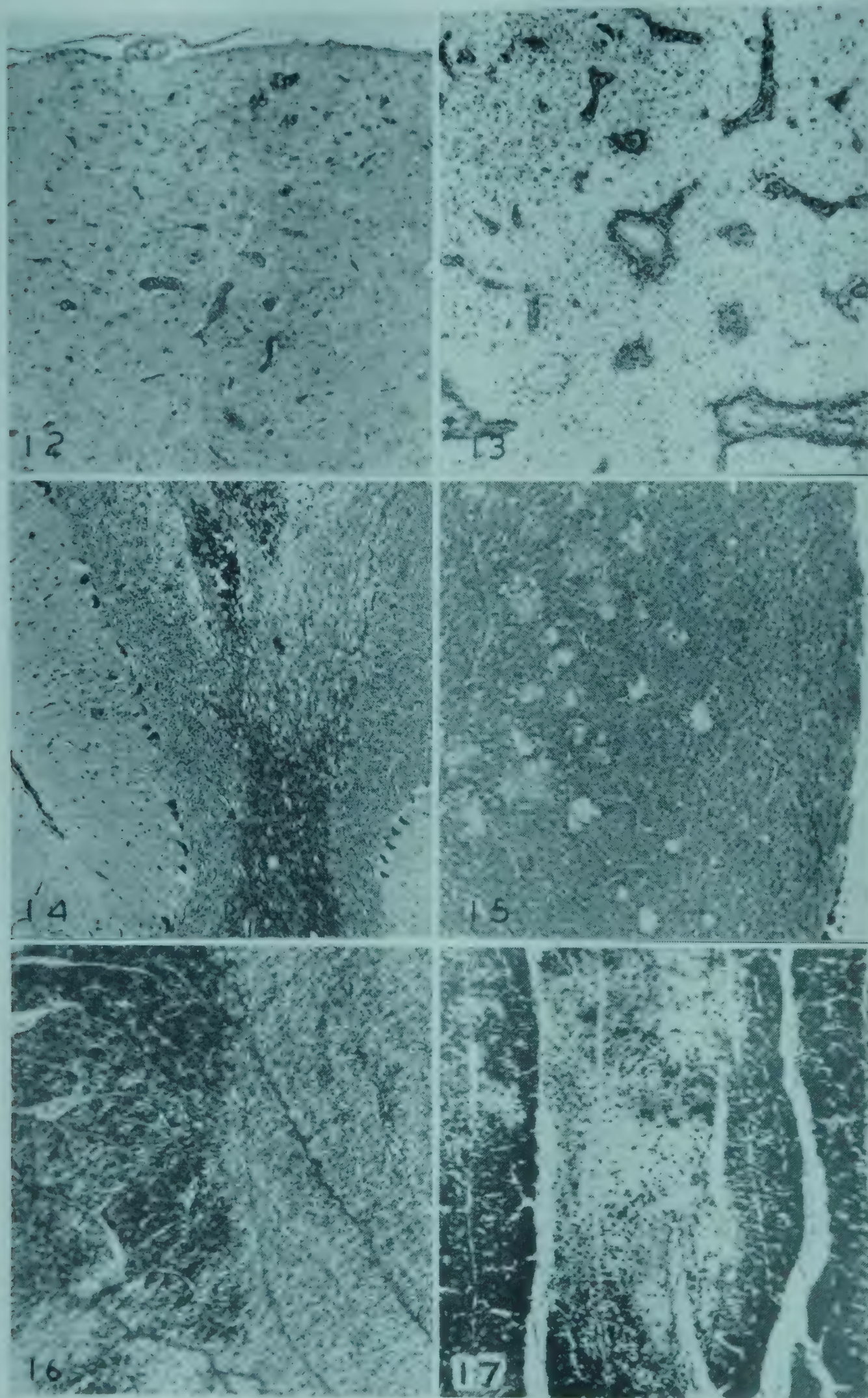


FIGURE 12-17 (*For description see facing page*)

All figures are photomicrographs of chickens affected with field encephalomalacia, stained with hematoxylin-eosin, except when stated otherwise. 80 X. (Slightly reduced).

Although the significance of the pinpoint lesions is not known, the fact that they are more common in the normal brain of the young than the adult bird suggests a connection with a maturation factor.

Inapparent Muscular Dystrophy in Pheasant. This change⁶ affects the anterolateral aspect of the breast muscle and consists of grayish fish-flesh-like areas or whitish striae. The lesions tend to occur in birds of either sex 8 to 18 weeks of age, but are more intense in the younger age groups. Histologically the lesion is characterized by Zenker's degeneration of affected muscular bundles and resembles the experimental condition in ducklings.²

Inapparent Ventricular Dystrophy in Chickens. In connection with the poultry meat inspection service, over 35 apparently healthy chickens of broiler age were observed to show grayish areas in the musculature of the gizzard, according to Brandly.⁷ The sections received showed predominantly hyaline necrosis of the smooth muscle fibers (FIGURE 16), accompanied by varying degrees of interstitial edema and sparse histiocytic and heterophilic infiltration. Other areas exhibited considerable cicatricial thickening of the interstices (FIGURE 17) and replacement fibrosis of the surrounding musculature, resulting in loss of architecture.

Summary

On the basis of the available laboratory diagnostic data, the incidence of acute field encephalomalacia from 1936 to 1947 ranged from 3 to 32 case lots, with an average of 18, per year. There were peaks in 1936 and in 1940 and there was a low in 1945. In 1948, the incidence was 62 known cases. The reasons for the yearly fluctuations were unknown. Feeding of a high energy-low fiber ration was not accompanied by an undue increase in 1947, but was in 1948. In the latter year, reduction of high fat-containing animal protein supplements in the diet seemed to bring about a decrease of the incidence.

Histopathologic observations on field specimens brought out: (a) that encephalomalacic foci in the brain may often be located outside the cerebellum and thus escape gross detection; (b) that extensive cerebellar fibrosis may occur with or without associated ischemic necrosis, the latter pathognomonic for the acute disease in chicks; (c) that the extracerebellar portions of the brain frequently show large areas of increased vascularity accompanied by varying degrees of adventitial cell proliferation; (d) that necrotic and reparative lesions are seen occasionally at one week of age and

FIGURES 12-17 (See opposite page)

FIGURE 12. 49 d.o. Cerebrum shows subpial focus of vascular and adventitial proliferation.

FIGURE 13. 126 d.o. Hypostriatum shows extensive vascular and adventitial proliferation and some intervascular gliosis.

FIGURE 14. 17 d.o. Cerebellum shows edema and hemorrhages indicative of acute field encephalomalacia, in upper portion of central white matter. Molecular layer in left center shows small glia foci indicative of avian encephalomyelitis. (Sections of pancreas, proventriculus, and other parts of brain confirmed latter diagnosis.)

FIGURE 15. 28 d.o. Same lot as FIGURE 9. Optic tract shows nonspecific unstained pinpoint areas. Mallory's phosphotungstic acid. (Section of nasal septum of this chick failed to exhibit microscopic evidence of A-hypovitaminosis.)

FIGURE 16. Approximately 84 d.o. Clinically inapparent myopathy of ventriculus. Dark staining normal smooth muscle tissue on left, hyaline necrosis of gizzard muscle on right. Masson's trichrome.

FIGURE 17. Same lot as FIGURE 16. Dark staining normal muscle tissue on both sides, light (green) staining scar tissue in center. Masson's trichrome. (Sections 16 and 17 by courtesy of Dr. Brandly.⁷)

thus suggest parentally transmitted deficiency; and (e) that reparative lesions may occur up to 32 weeks of age, alone or in association with other neuropathologic entities. Apparently recovered cases of field encephalomalacia may either escape detection or be diagnostically misleading.

Proved cases of field encephalomalacia have failed to exhibit evidence of vitamin A-hypovitaminosis by the method of nasal histopathology. Both spontaneous cases of field encephalomalacia and experimental cases of A-hypo- and hypervitaminosis showed achromatic pinpoint areas in the brain, which, therefore, were considered nonspecific for vitamin A deficiency.

Attention is called to the occurrence of clinically inapparent conditions resembling experimental E-avitaminoses in birds, namely: dystrophy of the voluntary muscle in pheasants, similar to that in ducklings, and dystrophy of the involuntary ventricular muscle in chickens, similar to that in poults.

The report brings out the relatively frequent occurrence in birds fed untreated natural feed stuffs of pathologic conditions reproducible by vitamin E-deficient diets. The occurrence of chronic lesions enhances the pathologic spectrum of field encephalomalacia.

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VITAMIN E DEFICIENCY, DIETARY FAT, AND SPINAL CORD LESIONS IN THE RAT*

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Introduction

Beginning with the studies of Goettsch and Pappenheimer,¹ much evidence has been presented showing that dystrophic changes in skeletal muscles are a characteristic manifestation of chronic vitamin E deficiency in laboratory animals. The lack of vitamin E in guinea pigs and rabbits causes a rapid onset of muscular dystrophy. This is considered a primary myopathy, since no changes have been found in the central nervous system, and since the degree and speed of recovery with vitamin E therapy is greater than would be expected if the central nervous system were damaged.

In rats, there is evidence that chronic vitamin E deficiency affects both the muscular and the nervous systems.² Neuromuscular lesions in the rat develop gradually after a period of about 5 months on experiment. Their response to vitamin E therapy is equivocal. Since the problem in the rat is more complex than in the guinea pig or rabbit, the question is raised as to whether the syndrome in the adult rat is primarily a neurogenic or a myogenic disease, or a combination of both.

Ringsted³ and Einarson and Ringsted² described four stages of clinical symptoms in the chronic E-deficient rat. These stages are characterized by progressive motor and sensory disturbances with concomitant muscular atrophy. They reported that the initial lesion in the central nervous system was a degeneration in the lumbar cord, affecting the proximal parts of the posterior roots and the proprioceptive paths in the posterior columns. The amount of neuroglial reaction was inconstant. Degeneration of the anterior horn cells of the lumbar cord generally began shortly after the degeneration of the posterior columns. Other workers^{4,5} have also described degeneration of the central nervous system in chronic E-deficient adult rats. De Gutiérrez-Mahoney⁵ reports much more widespread damage to the central nervous system than previous investigators.

These experimental findings led to numerous clinical studies on the use of vitamin E in the treatment of amyotrophic lateral sclerosis and other chronic degenerations of the nervous system. The results, which were contradictory and largely negative in nature, have been summarized by Wolf and Pappenheimer,⁶ who conducted a restudy of the nervous system in chronic vitamin E-deficient rats. They concluded that, under the experimental conditions obtaining in their laboratory, lesions of the central nervous system did not occur. It is their opinion that the lesions previously described were due to some factor in the experimental procedure other than the lack of vitamin E.

Because of these differences in experimental findings we have carried out

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the studies outlined in the following in an effort to re-evaluate the effects of chronic vitamin E deficiency on the central nervous system of the rat, and to test whether the state of oxidation of fat in diets used may modify the results obtained.

Materials and Methods

Albino rats from normal stock breeders were placed on experimental diets on the 21st–35th day of life. The dietary constituents were as follows:

Vitamin-free casein	20%
Cerelose	55%
Dried yeast	7%
Salt mixture No. 2 (U.S.P.)	3%
Lard (type varied, see TABLE 1)	15%
Vitamin A	400 I.U. } given orally
Vitamin D	40 I.U. } twice weekly.

Ten different dietary groups were arranged, as indicated in TABLE 1. Control rats were given, twice weekly by pipette, 15 mg. of a concentrate

TABLE 1

COMBINATIONS OF LARD, VITAMIN E, AND ESSENTIAL FATTY ACIDS (FA) IN DIETS USED AND NUMBER OF ANIMALS STUDIED

<i>Lard variations</i>	<i>Combinations</i>		<i>No. animals</i>	
	<i>Control</i>	<i>Experimental</i>	<i>On exper.</i>	<i>C.N.S. studied</i>
Ordinary commercial lard (L)	L + E		4	3
		L – E	3	3
Commercial lard oxidized for 12 hrs. by bubbling	OL + E		5	4
		OL – E	4	3
4 liters of air per minute through lard heated to 100 C. (OL)	OL + FA + E		6	3
		OL + FA – E	10	0
Commercial lard oxidized for 24 hrs. by bubbling	SL + E		4	0
		SL – E	12	1
4 liters of air per minute through lard heated to 100 C. (SL)	SL + FA + E		4	2
		SL + FA – E	12	1

of mixed natural tocopherols* (containing 34 per cent tocopherols, of which approximately one-half was in the form of alpha-tocopherol). Two groups of animals were given methyl esters of corn oil (0.1 cc., twice weekly) to compensate for a possible destruction of essential fatty acids in the oxidized

* The tocopherol concentrate and the concentrate of vitamin A and D, were kindly supplied by Distillation Products, Inc., Rochester, N. Y.

lard. The diets were freshly prepared every 10 to 14 days, and were kept refrigerated.

During the course of this investigation, 64 animals were maintained on the diets for varying lengths of time. Of these, 44 rats died from apparent toxicity of certain diets, especially those containing the lard oxidized for 24 hours. A few showed evidence of respiratory or other intercurrent infections. It was found that young rats from litters in which mother and young were reared throughout lactation and to the 30th day of age on a semi-synthetic diet containing no added fat were much more resistant to the toxic effects of the oxidized-fat diets than were young from litters on the stock diet put on experiment at 21 days of age.

Twenty rats (12 controls, 8 deficient) were sacrificed at approximately 9-10 and 12 months of age. Eight of the animals (4 -E and 4 +E) were fixed by injecting 10 per cent neutral formalin into the spinal canal. Twelve of the animals (4 -E and 8 +E) were fixed by perfusion through the aorta with normal saline followed by neutral formalin. The carcasses were then skinned and stored in 10 per cent neutral formalin for about three months. The solution was changed twice in the interval. The cranial vaults were then opened, the spinal cords exposed by laminectomy, and the carcasses stored for several months in 10 per cent neutral formalin. Segments of the cord and spinal ganglia were removed from the cervical, thoracic, and lumbar regions. The forebrain, midbrain, and hindbrain were removed in a single piece.

Paraffin, frozen, and celloidin sections (cut at 10, 20, and 25 microns, respectively) were prepared from the cerebrum, cerebellum, spinal cord, and spinal ganglia of the rats. Representative sections from the cervical, thoracic, and lumbar regions were stained by the following methods: Weigert-Pal, Weil's method,⁷ and the rapid myelin method of Smith and Quigley⁸ for the study of myelin sheaths; galloxyanin for nerve cells, axons, and Nissl substance; Kinyoun's⁹ carbol-fuchsin stain for acid-fast pigment; Sudan IV for neutral fat. Sections of cerebrum, cerebellum, and spinal ganglia were stained by the galloxyanin and Smith-Quigley methods. Thoracic and abdominal viscera and skeletal muscle from all animals were fixed in Zenkers solution (after the formalin injections), imbedded in paraffin, and stained with Kinyoun's acid-fast stain and with hematoxylin and eosin.

Results and Discussion

(1) *Gross Manifestations.* The onset of evident paresis in the vitamin E-deficient rats is recorded in TABLE 2. The subsequent development of neuromuscular disturbances was a slowly progressive one, following essentially the same sequence as described by Einarson and Ringsted.² Among the first symptoms noted were hyperkinesia and tremors and hypalgesia. The latter was especially evident in the course of periodic blood studies on these animals, which necessitated cutting the tip of the tails for blood specimens. Compared to the control animals, all the E-deficient rats in Ringsted's stage I or beyond reacted sluggishly to this procedure.

Comparison of the growth curves of vitamin E-deficient rats and those

TABLE 2

VITAMIN E-DEFICIENT RATS: AGE OF ONSET OF PARESIS AND AGE AND CLINICAL STAGE OF PARESIS AT TERMINATION

<i>Exp. group</i>	<i>Rat No.</i>	<i>Age at onset of paresis (weeks)</i>	<i>Age at termination (weeks)</i>	<i>Clinical stage of paresis (Ringsted)</i>
L — E	4504	21	40	II
	4510	21	52	III
	4520	21	52	III
OL — E	4506	27	40	II
	4516	27	41	II
	4512	28	52	III
SL + FA — E	4568	28	40	II
SL — E	4561a	—	43	O — I

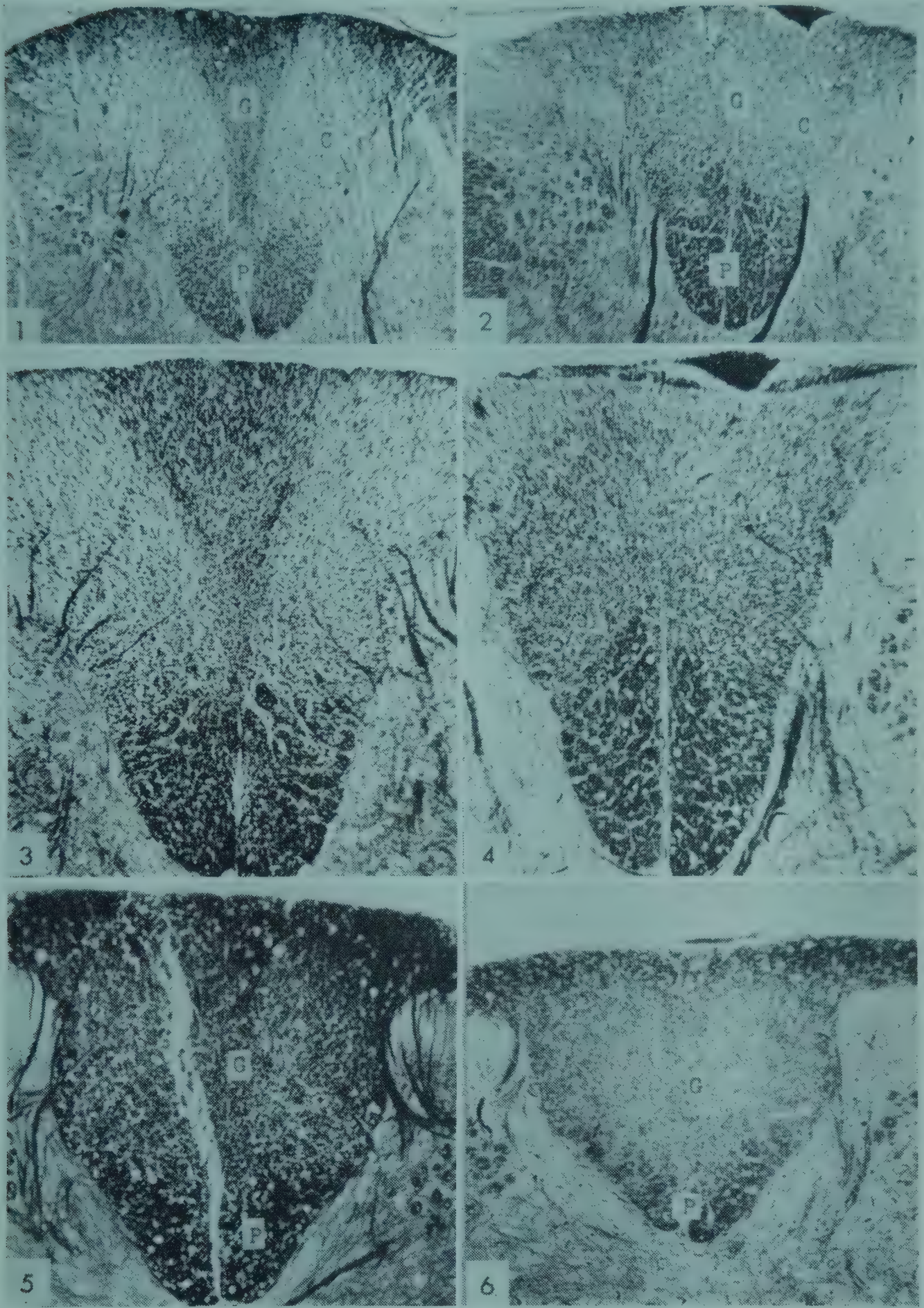
receiving oral vitamin E supplements clearly show that both groups followed similar trends of growth until about the third month. Beginning at about this time, the growth of the vitamin E-deficient rats reached a plateau, while that of those receiving tocopherols continued at a normal rate.

The oxidation products of the unsaturated fatty acids of commercial lard (viz. peroxides, aldehydes, ketones, and further decomposition of reactive peroxides by mechanisms of polymerization and splitting), many of which may be quite unphysiological, undoubtedly account for the toxic symptoms shown by the rats fed vitamin E-deficient diets containing oxidized fatty acids. Whipple¹⁰ has found that dogs fed on a diet containing oxidized fat developed a disease which she termed the “oxidized fat syndrome.” These dogs showed loss of hair, skin lesions, anorexia, emaciation, and intestinal hemorrhages. The experiment was subsequently repeated on rats with similar results.¹¹ It is to be noted that in these studies no provision was made for a dietary source of vitamin E.

As indicated in TABLE 1, rather marked toxicity was shown by rats fed three of the vitamin E-deficient diets, namely, those containing: (a) lard oxidized for 12 hours and essential fatty acids; (b) lard oxidized for 24 hours; and (c) the same, supplemented with essential fatty acids. These rats

FIGURES 1-6 (See opposite page)

- FIGURE 1. Control rat, cervical cord, showing normal myelination of the fasciculus gracilis (G), fasciculus cuneatus (C), and pyramidal tract (P). From rat of L + E group, age 366 days. Weil stain. X 40.
- FIGURE 2. Deficient rat, cervical cord, showing marked reduction of myelin-sheath substance in the fasciculus gracilis (G) and loss of demarcation between the fasciculi gracilis and cuneatus (C). The pyramidal tract (P) is intact. From rat of OL-E group, age 277 days. Weil stain. X 40.
- FIGURE 3. Control rat, description the same as for FIGURE 1. Rat from OL + E group, age 467 days. Smith-Quigley rapid myelin stain. X 60.
- FIGURE 4. Deficient rat, description the same as for FIGURE 2. Rat from L-E group, age 276 days. Smith-Quigley rapid myelin stain. X 60.
- FIGURE 5. Control rat, lumbar cord, showing normal appearance of fasciculus gracilis (G) and pyramidal tract (P). The vertical streak in the section is an artifact. From rat of SL + FA + E group, age 365 days. Weigert-Pal stain. X 60.
- FIGURE 6. Deficient rat, lumbar cord, showing marked reduction of myelin-sheath substance in the fasciculus gracilis (G). Pyramidal tract (P) is intact, though not well shown. From rat of L-E group, age 365 days. Weigert-Pal stain. X 60.

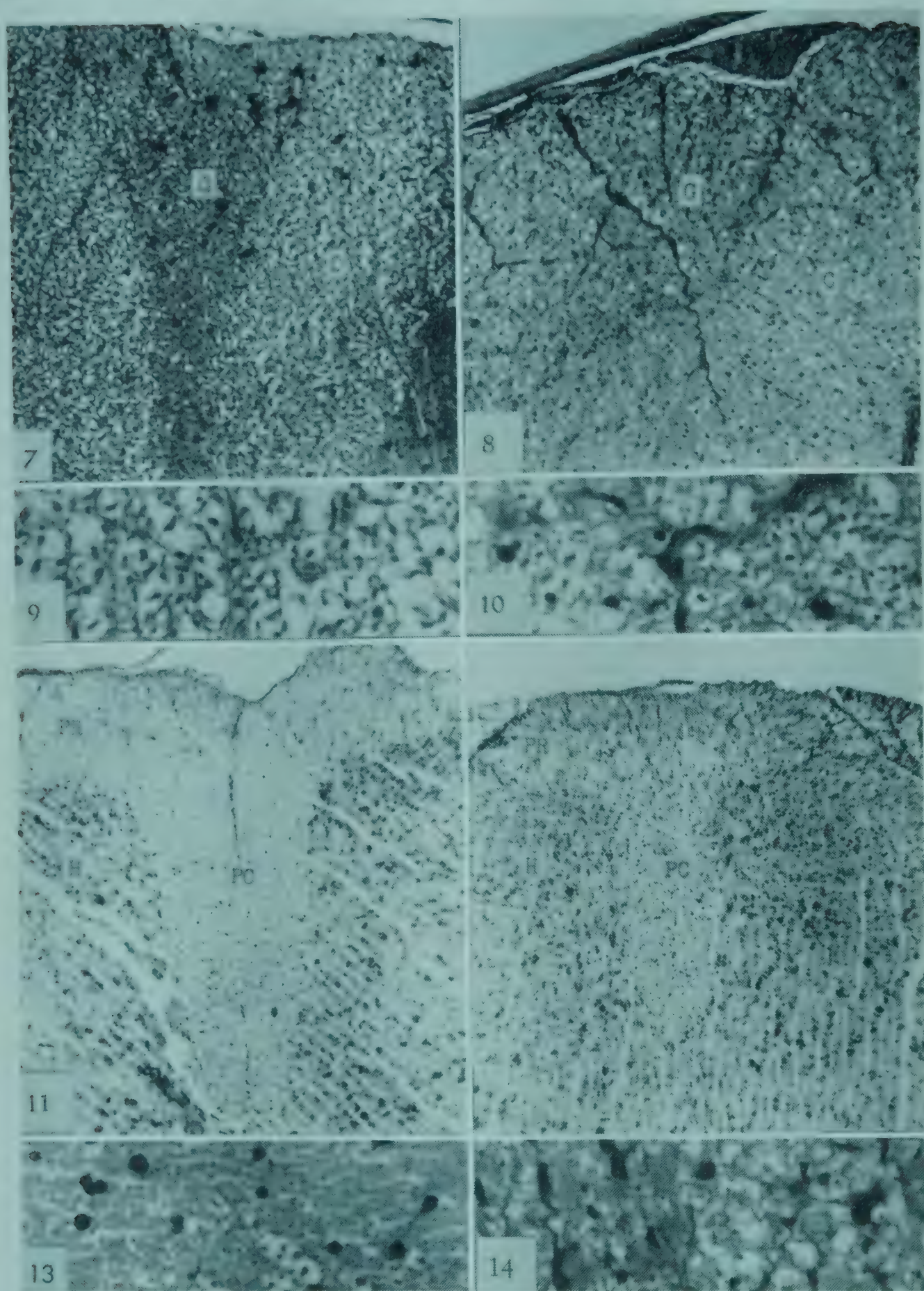


FIGURES 1-6 (*For description see facing page*)

Photomicrographs of transverse sections through the dorsal portions of the spinal cords of control (left) and vitamin E-deficient rats (right), at the level C5 of the cervical enlargement (FIGURES 1 to 4) and level L4 of the lumbar enlargement (FIGURES 5 and 6), stained for myelin by three different methods.

showed progressive symptoms of asthenia, anorexia, alopecia and loss of hair luster, and a moderate to intense diarrhea. Many succumbed during the 2nd and 3rd months of experiment. It was surprising to find that such small amounts of methyl esters of corn oil (0.1 cc., twice weekly) should definitely aggravate the deleterious effects of oxidized fats in the deficient diets.

It is also significant that rats given oral supplements of vitamin E showed



FIGURES 7-14 (For description see facing page)

FIGURES 7-14 (See opposite page) Photomicrographs of transverse sections through the dorsal portions of the spinal cords of control (left) and vitamin E-deficient rats (right), at level C5 of the cervical enlargement (FIGURES 7 to 10) and level L3 of the lumbar enlargement (FIGURES 11 to 14), stained with gallocyenin.

excellent growth and vigor and no evidence of toxic reactions, when reared on diets containing oxidized lard, with or without supplements of essential fatty acids. Even though a portion of the tocopherol may have been destroyed by lard-oxidation products in the gastrointestinal tract, sufficient vitamin was absorbed to maintain normality.

Neuromuscular disorders invariably appeared earlier in rats fed vitamin E-deficient diets containing fresh lard than in those containing lard oxidized for 12 hours, which is in accord with similar observations by Einarson and Ringsted.² Of particular interest is the fact that the appearance of paresis

was even more delayed with diets containing lard oxidized for 24 hours, despite the toxic reactions and general debility shown by animals fed such diets.

(2) *Histological Observations.* In the animals surveyed in this study, there was no constant evidence of degenerative changes in the motor cells of the anterior horn or in cells of the posterior horns. Hyperchromatosis was observed inconstantly and with equal frequency in both control and experimental animals. The abnormal changes characterizing the vitamin E-deficient animals were primarily those of demyelination of localized zones and associated glial reactions. Dystrophic changes, identical to those described by Einarson and Ringsted² and many other investigators, were found in all the deficient rats. The extent of these changes was roughly proportional to the neurologic lesions described below.

Frozen, paraffin, and celloidin sections of spinal cord stained by three myelin sheath stains (Weil,⁷ Smith-Quigley,⁸ and Weigert-Pal methods) showed degenerative changes in the posterior columns and in the proximal parts of the posterior roots. There was a consistent reduction of myelin sheath substance in the posterior sensory columns and proximal parts of the posterior roots in all of the chronic vitamin E-deficient rats. This reduction of myelin sheath substance was present in both the cervical (compare FIGURES 1 and 3 with FIGURES 2 and 4) and lumbar (compare FIGURES 5 and 6) cords. The pyramidal tracts in all of the animals were completely intact.

Sections stained with gallocyannin consistently showed degenerative changes in the posterior sensory columns and in the proximal parts of the posterior roots of E-deficient animals but none in the controls. In the control animals, the axons of the posterior columns were arranged in an orderly fashion and were surrounded by a clear zone represented by the myelin sheaths. These, in turn, were circumscribed by the delicate, lacelike processes of the neuroglial cells (FIGURES 7, 9, 11 and 13). On the other hand, in all the deficient rats, a moderate to marked reaction of gliosis was observed in the posterior columns and in the proximal parts of the posterior roots (FIGURES 8, 10, 12, and 14). This was more apparent in the fasciculus gracilis (FIGURE 14) but was also present in the fasciculus cuneatus (FIGURE 10). The glial pattern appeared as an irregular coarse network. This, in turn, produced a distortion of the usual orderly axon arrangement. The extent and type of degeneration was essentially of the same magnitude in both lumbar and cervical segments

FIGURE 7. Control animal, cervical cord, showing the fasciculus gracilis (G) and cuneatus (C). The pyramidal tract is not shown. The axons (ensheathed by unstained rings of myelin) and the glial cells are darkly stained. Rat of L + E group, age 365 days. $\times 80$.

FIGURE 8. Deficient rat, cervical cord, showing extensive gliosis in the fasciculi gracilis (G) and cuneatus (C). The pyramidal tract is not shown. Note that the architecture of the posterior sensory column is markedly altered, and also that there is a reduction in the size of the unstained ensheathing rings of myelin. Rat from L-E group, age 276 days. $\times 80$.

FIGURE 9. Higher magnification of an area in the fasciculus cuneatus of FIGURE 7. Note the axons ensheathed by unstained rings of myelin and the units demarcated by delicate, lace-like glial processes. $\times 320$.

FIGURE 10. Higher magnification through the fasciculus cuneatus of FIGURE 8. Note the reaction of gliosis, reduction of unstained rings of myelin sheath substance, and the coarse glial pattern. $\times 320$.

FIGURE 11. Control rat, lumbar cord, showing the posterior column (PC) and the proximal parts of the posterior roots (PR), composed of densely packed axons surrounded by clear rings of myelin, and the dark circular glial cells. To the right and left are the borders of the posterior horns (H). Rat of L + E group, age 365 days. $\times 80$.

FIGURE 12. Deficient rat, lumbar cord, showing extensive glial proliferation in the proximal parts of the posterior roots (PR) and in the posterior sensory columns (PC). To the right and left the borders of the posterior horns (H) are shown. Rat of OL-E group, age 277 days. $\times 80$.

FIGURE 13. Higher magnification of the fasciculus gracilis in FIGURE 11. Note the closely packed axons and the uniformity in shape of the glial cells and the glial pattern. $\times 320$.

FIGURE 14. Higher magnification of the fasciculus gracilis of FIGURE 12. Note especially the gliosis, and the distortion and coarseness of the axon pattern. $\times 320$.

The neuropathologic lesions were less extensive and less severe in rats fed the diets containing oxidized lard than in those receiving the diets with fresh lard for comparable periods of time. This is in keeping with the differences observed in the onset of paresis in these experimental groups. It is also worthy of note that the degenerative changes and pigment accumulation in skeletal, cardiac, and smooth muscle characteristic of vitamin E-deficient rats¹² were least marked in rats fed oxidized lard, especially that treated for 24 hours, and were most marked when fresh lard was incorporated in the diets.

Summary

Rats reared on vitamin E-deficient diets showed, after 5 to 7 months of age, increasing evidence of hypalgesia and progressive paresis. When sacrificed at 9 to 12 months of age, the posterior columns (fasciculi cuneatus and gracilis) and proximal parts of the posterior roots of the cervical, thoracic, and lumbar segments of the spinal cord consistently showed evidence of demyelination, gliosis, and distortion of the axon pattern. No significant alterations were observed in the pyramidal tract, the cerebellum, or the cerebrum. These findings generally confirm earlier observations of Einarson and Ringsted and others.

When lard oxidized for 12 or for 24 hours was used in place of fresh lard in the experimental diets, the onset of paresis was delayed, the severity of the neurologic lesions diminished, and the characteristic alterations in skeletal and other musculature were less marked. Yet, the oxidized lard, especially that treated for 24 hours, was not well tolerated by the rats simultaneously deprived of vitamin E. No such reactions occurred when oral supplements of tocopherol were given.

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UTERINE AND VAGINAL CHANGES IN RATS WITH AVITAMINOSIS E

II. COLLAGENOUS FIBERS CONTENT OF THE ENDOMETRIUM OF NORMAL RATS AT DIFFERENT AGES*

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It was reported in a previous paper¹ that in vitamin E-deficient rats a fibrosis of the endometrium takes place. It was not ascertained, however, whether this phenomenon was due to the vitamin deficiency itself or related to the advanced age of the animals.² The elucidation of this point is very important, because such a fibrosis may be responsible for the failure of implantation reported in vitamin E-deficient animals^{3, 4, 5} and for the irreversible sterility observed in prolonged avitaminosis E.⁶ In order to study further the nature of the above mentioned fibrosis, an investigation was made of the endometrium of normal rats, from our colony, at different age periods.

Material and Technique

Sixty-seven female rats, receiving a basic diet supplemented with peanut or germinated maize, were killed at the ages 9 to 12, 30, 60, 116, and 150 to 240, 390, 450 to 690 days. Each group, but the latest (7 rats), contained 10 animals. Fixation of the uteri was in 10% formalin. From each animal a median piece of both uterine horns was taken and embedded in paraffin according to usual technique. Sections were stained by hematoxylin-eosin and van Gieson.

Results

Macroscopically, the rat uteri of all groups were normal. It is not my intention to describe in detail the histologic appearance of the rat's endometrial stroma, because it has been done before by Wolfe *et al.*² I will report only its content of cells and collagenous fibers in the several animal groups.

Endometrium of 9 to 12 Day Rats. The stroma was very cellular, presenting no collagenous fibers, or showing, in a few areas, near the myometrium, either a reddish intercellular substance (van Gieson staining) or very thin and inconstant collagenous fibers.

Endometrium of 30 Day Rats. The stroma presented few and thin collagenous fibers occupying approximately the third part (6 cases) or one half (4 cases) the thickness of the endometrium, situated near the myometrium. The other part of the endometrium did not contain collagenous fibers (FIGURE 1).

Endometrium of 60 Day Rats. The appearance of the stroma was similar to that of 30-day rats, but the collagenous fibers were more numerous, occupying about two-thirds (1 case), three-fourths (5 cases), or even all the thickness of the endometrium.

* I wish to thank Dr. Dutra de Oliveira for his aid in this work.

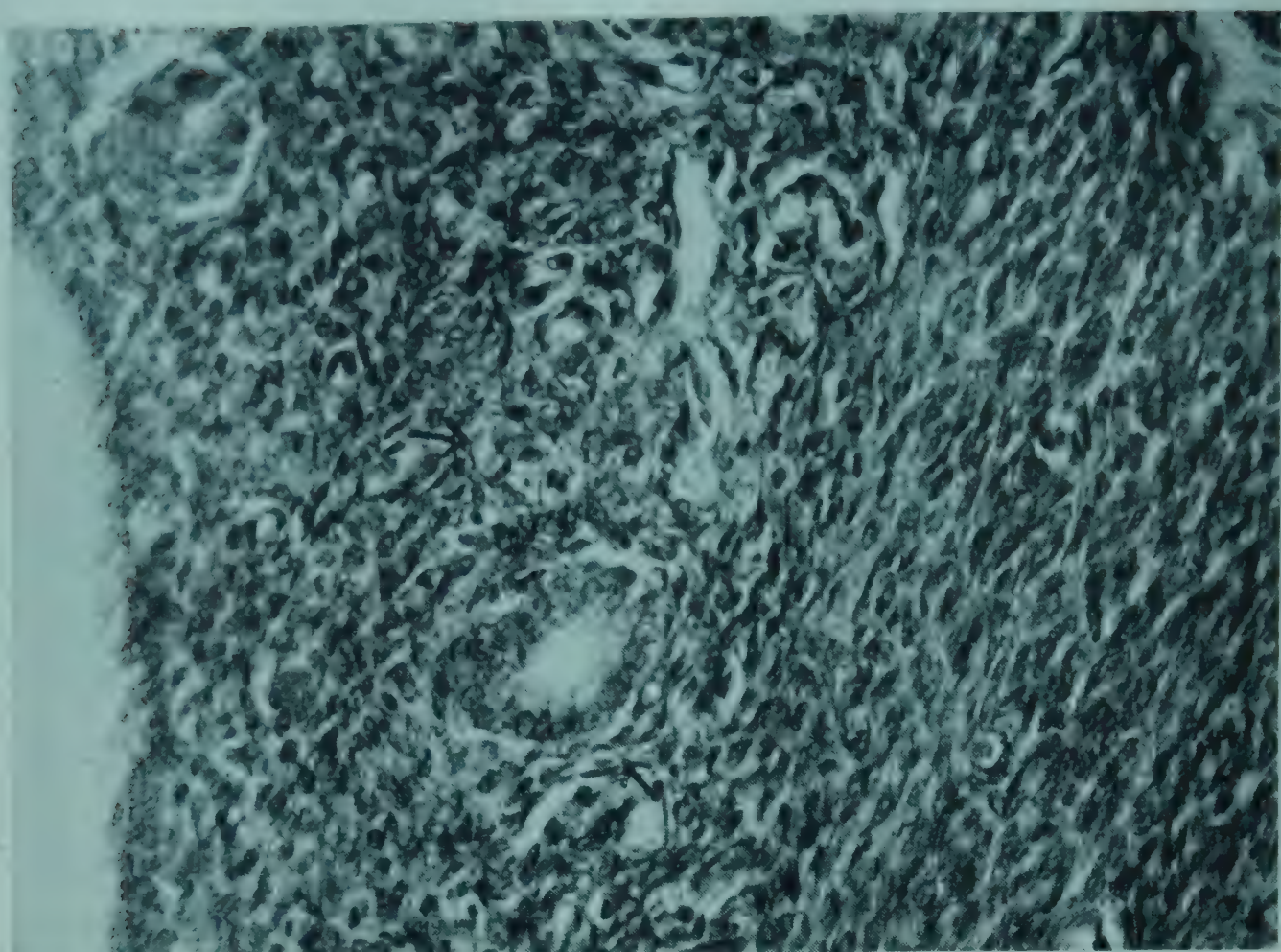


FIGURE 1. Thirty-day-old rat. Note the great cellularity of the endometrium (left), with few inconspicuous collagenous fibers (arrows) in only the outer half near the myometrium (right). Hematoxylin-van Gieson staining. $\times 400$.

Endometrium of 116 Day Rats. The stroma was richer in collagenous fibers than in the previous group, containing an average number of such fibers. These fibers were thicker and occupied about four-fifths (8 cases) and, rarely (2 cases), three-fourths of the thickness of the endometrium. The remaining endometrium, bordering the uterine lumen, did not present collagenous fibers.

Endometrium of 150 to 240 Day Rats. The stroma had the same appearance as that of 116-day rats. The collagenous fibers, however, occupied five-sixths (9 cases) and, exceptionally, two-thirds of the thickness of the endometrium (FIGURE 2).

Endometrium of 390 Day Rats. The stroma had a changing content of collagenous fibers. In 2 cases, it presented thin collagenous fibers, occupying only one-third of the thickness of the endometrium, adjacent to the myometrium. In 4 cases, the stroma was like that of 150 to 240 day rats. In the four remaining cases, the stroma contained collagenous fibers of mean and great thickness, occupying about five-sixths of the thickness of the endometrium.

Endometrium of 450 to 690 Day Rats. The stroma was rich in very thick collagenous fibers, occupying about nine-tenths (5 cases) and, rarely (2 cases), four-fifths of the thickness of the endometrium, only the part bordering the endometrial lumen remaining free of collagen (FIGURE 3). Careful examination of the myometrium revealed no pigment (lipofuscin).

TABLE 1 presents the content of cells and collagenous fibers of the endometrial stroma of rats of different ages.

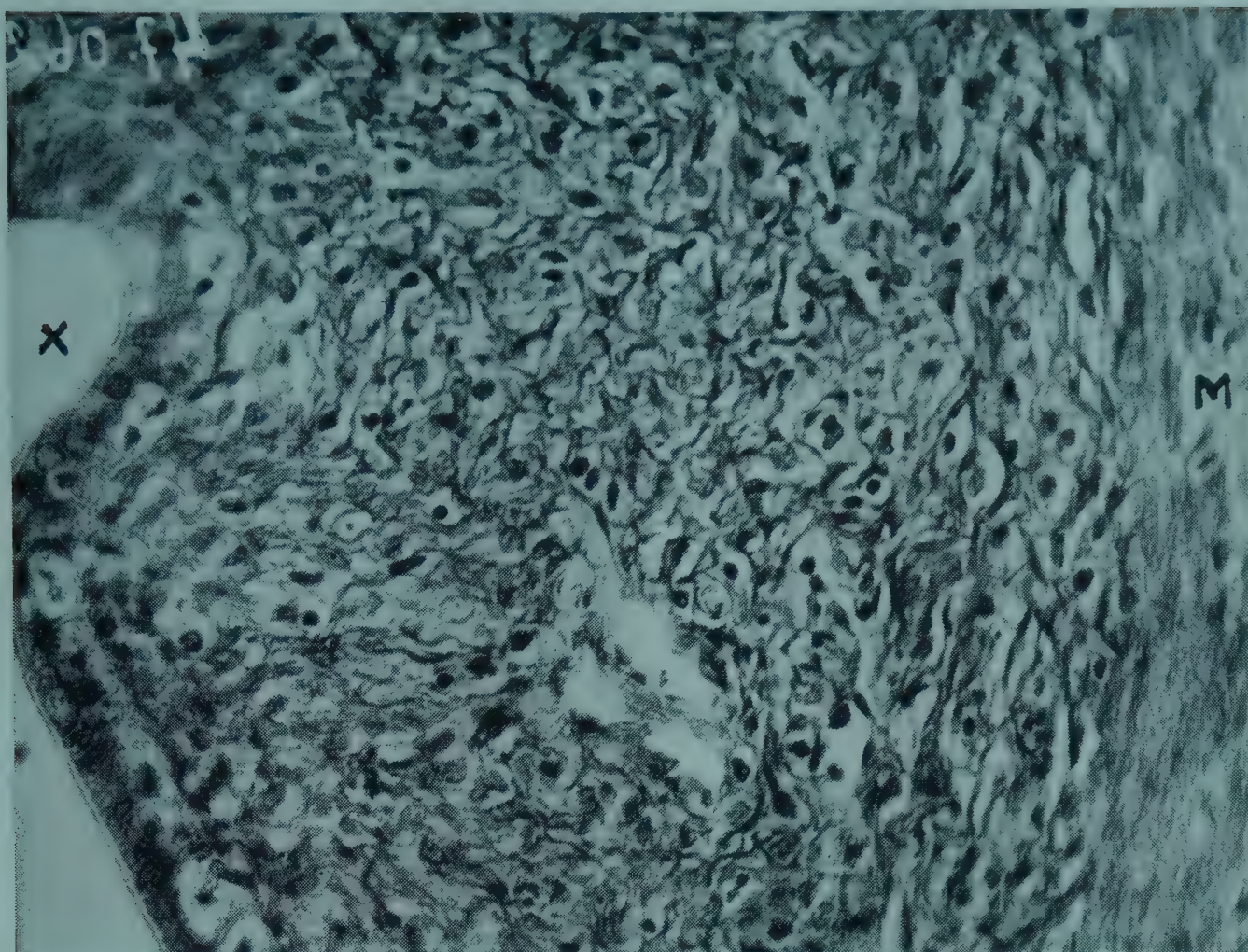


FIGURE 2. Two hundred and ten-day-old rat. The endometrium contains more conspicuous and abundant collagenous fibers than in preceding figure. Uterine lumen (x) at the left and myometrium (M) at the right. Hematoxylin-van Gieson staining. $\times 400$.

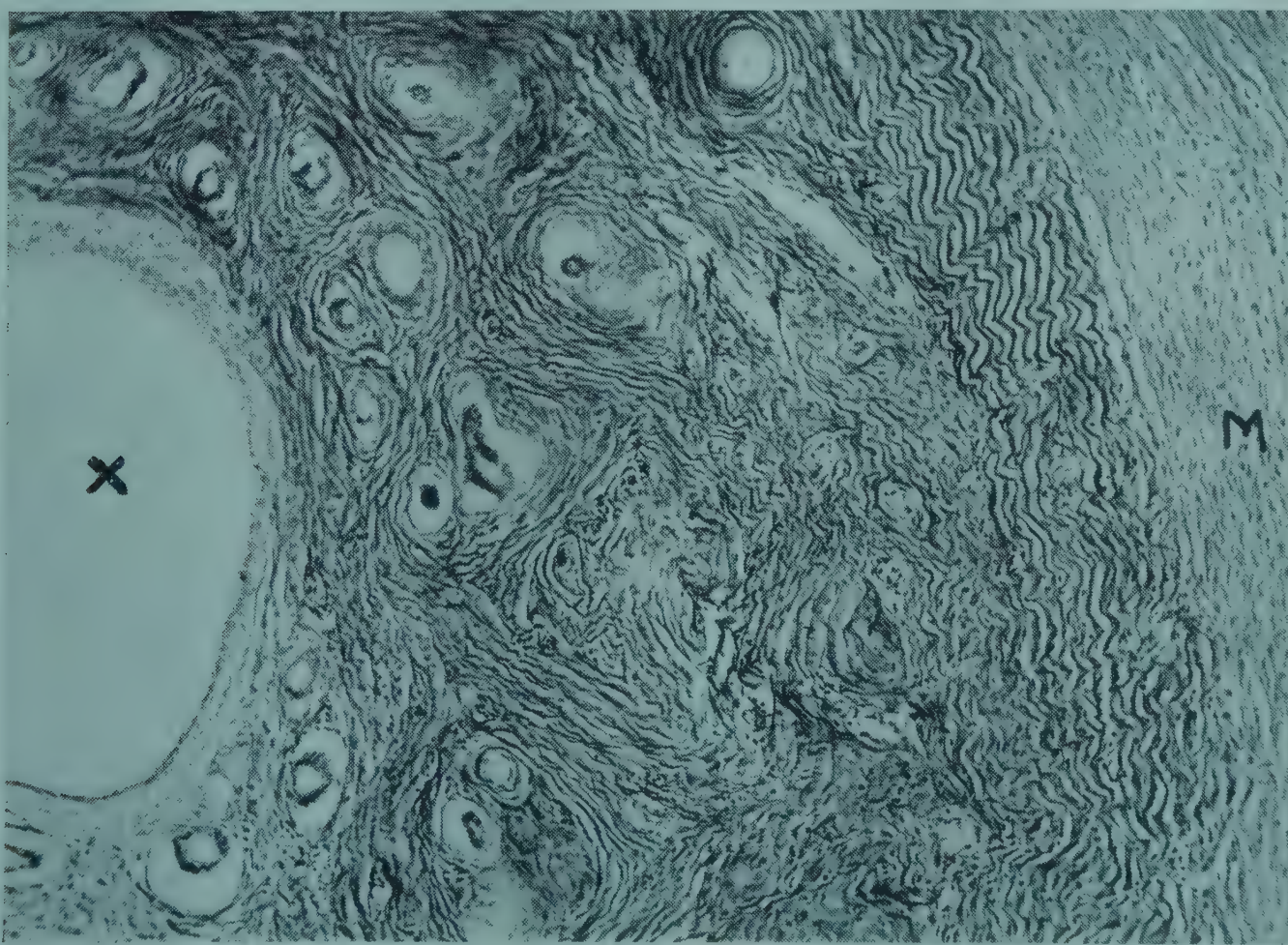


FIGURE 3. Six hundred and ninety-day-old rat. Note the enormous number and thickness of the collagenous fibers of the endometrium. Uterine lumen (x) and myometrium (M) are shown. Hematoxylin-van Gieson staining. $\times 80$.

TABLE 1

Age in days	Stromal cells	Collagen	Observations
9 to 12	++++	±	No collagenous fibers; or inconstant and very thin collagenous fibers.
30 to 60	+++	+	Thin collagenous fibers.
116, 150 to 240 and 390	++	++	Thin collagenous fibers.
390, 450 to 690	+	+++	Collagenous fibers of mean and great thickness.

Discussion

I have confirmed, in the rats of our colony, the results of Wolfe *et al.*,² namely that there is, accompanying advancing age in the animals, with a few exceptions (2 animals in the group of 390-day rats), a progressive increase of the collagen of the rat endometrium. Therefore, the endometrial fibrosis reported in the first part of this paper¹ is a physiological fact, related to the advanced age of the animals and not due to the vitamin E deficiency.

Summary and Conclusion

The author, in order to elucidate whether the endometrial fibrosis observed in rats with avitaminosis E is due to the vitamin deficiency or to the advanced age of the animals, studied the endometrium of rats from 9 to 690 days old. He noted, confirming the reports of other authors, that there is a progressive increase of the endometrial collagen with advancing age in the animals. On the basis of comparisons made between the uteri of vitamin E-deficient rats, previously described, and those of normal rats of comparable ages included in the present study, it was concluded that this fibrosis of the deficient animals was related to the advanced age of the animals and not due to the vitamin E deficiency.

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CEROID SUBSTANCE AND ITS MEANING

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The biological action of vitamin E extends beyond the sexual sphere into more general phases of nutrition. Consequently, vitamin E must be thought of as participating in a successive series of metabolic reactions in the absence of which multiple phenomena may occur. It may be regarded as a component of a biological system and not merely as an antioxidant.

We have had the opportunity of studying, with Lopes de Faria, metabolic disturbances in vitamin E-deficient animals exhibiting muscular dystrophy and the accumulation of ceroid substance. The latter material, extensively studied by other workers, seems related to the formation of peroxides, and consists of a recently formed liposoluble part and an insoluble component of lipo-proteic nature. Histologic changes of a necrotic and degenerative type occur gradually, associated with the appearance of macrophages and fibroblasts. Biochemically, lipid metabolism, especially that related to the utilization of unsaturated fatty acids, must be gradually altered.

The utilization of carbohydrate and fat for the production of energy requires that catalyzers concerned in cellular respiration perform a series of reactions leading hydrogen to cellular oxygen in the continuous phases of oxy-reductions. The participation of riboflavin and of nicotinamide in carbohydrate metabolism, and of thiamine more specifically in pyruvate metabolism, are significant examples. Pyruvic acid is, in turn, related to the metabolism of proteins (alanine) and of fatty acids.

In the experimental conditions under discussion, there are disorganized phosphorylations to which the protein-glyco-lipid metabolism is subjected, leading to disharmony in the breakdown of fatty acids, with polymerization of peroxides of lipoprotein type which are more stable than the unsaturated fatty acids composing them.

Metabolic dysfunctions of lipids leading to the formation of ceroid occur in experimental vitamin E deficiency. Here, attention should be given to abnormalities of lipid metabolism, especially if there is a concomitant hypoproteic regimen. The questions of nervous influences and of acetylcholine metabolism, and the interference of thiamine in balanced relationships in distribution of cholinesterases and of acetylcholine leading to increased vagal actions, also warrant consideration.

We have had the opportunity to study the influence of hypoproteic and hyper-fat diets on lipid fractions of the liver. The results were as follows:

<i>Diets</i>	<i>Free cholesterol mg. %</i>	<i>Cholest. esters mg. %</i>	<i>Total choles. liver</i>	<i>Fatty acids mg. %</i>	<i>Phospho- lipids mg. %</i>
Normal	3.70	5.81	7.68	180.5	111.6
Hypoproteic and hyperfat	3.89	12.63	16.44	282.8	211.7

In these animals, the regime of which was low in vitamin E, we observed the same phenomena as in those subjected to experimental vitamin E deficiency.

FLUORESCENT PIGMENTS IN THE UTERI OF VITAMIN E-DEFICIENT RATS

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Various authors have described, in vitamin E-deficient rats, the accumulation of lipo-fuscin pigment, which is fluorescent and takes an orange stain with Sudan III, similar to that of other lipoids. Since it can be demonstrated in paraffin sections, one may deduce that the pigment is insoluble in fat solvents, and, because of these characteristics, it has been classed as "ceroid." This pigment stains intensely with fuchsin, retaining its color more tenaciously than other tissue elements. When the sections are stained by the Gallego method (Ziehl-picro indigo carmin), the nuclei are stained with fuchsin, and the pigment appears as brilliant red granules.

The pigment is fluorescent and, when exposed to ultraviolet light filtered through Wood glass (3650 Å), yields a characteristic primary clear yellow fluorescence.

This lipo-fuscin pigment has been found by various authors in avitaminosis E in the muscular coat of the uterus, in the vaginal musculature, and within skeletal muscle fibers.

Material. Rats in a state of vitamin E deficiency

- (1) 2 animals received daily injections of 1 mg. of progesterone.
- (2) 2 daily with 1 mg. of alpha-tocopherol by mouth.
- (3) 2 maintained as controls without supplement.

Methods. The studies were made using a 125 watt mercuric vapor lamp (Type "Philora") with Wood Corning glass selector filter. The microscope was of the usual type with apochromatic objectives. The ocular was equipped with a protective filter (Type "Euphos"). Ordinary slides and cover glasses were used. Frozen sections and paraffin sections (the latter deparaffinized) were mounted in water, glycerine or in Shillaber's immersion oil, or in a solution of polystyrene in xylol. The last medium has given the best results because it has very slight primary fluorescence and great transparency to ultraviolet radiation, giving images of extreme clarity and definition. With the evaporation of the xylol, the substance solidifies and yields a permanent mount after 24 hours.

Macroscopic studies were made with an ordinary commercial lamp of the brand designated above.

Macroscopic Fluorescence. Vitamin E-deficient rats, when examined with the Wood lamp at 3650 Å show whitish fluorescence of the panniculus adiposus which had lost its normal yellow fluorescence—a phenomenon noted in previous studies of rats with avitaminosis A. The lack of characteristic fluorescence in the subcutaneous fat may thus be related to lack of vitamin A in the tissue of rats deprived of vitamin E. In the liver, one noted a clear greenish fluorescence less intense than is found in normal animals. This also is due to a progressive deficiency of vitamin A.

The uterus in ordinary light is of an intense chestnut color and, with

the Wood lamp, exhibits deep yellowish luminescence due to the lipo-fuscin pigment accumulated in this organ.

Fluorescent Microscopy. The uteri, fixed in 10 per cent formalin, were cut with the freezing microtome or embedded in paraffin. Some of the frozen sections were preserved in formalin for 6 months, and then examined.

Both frozen and paraffin sections exhibit diffuse primary fluorescence and, in addition, the fluorescence typical of the lipo-fuscin pigment which is found in medium-sized granules within the muscle fibers of the uterus, both in the internal and external layers. In some sections, the pigment is more abundant in the outer zone, as noted by de Faría. In the fibrous submucous tissue of some of the atrophic uteri, and between the two muscular coats, are aggregations of macrophages laden with pigment in variable numbers. Occasionally there were found isolated elements, with only a small quantity of pigment occurring as scattered granules.

Mounted frozen or paraffin sections conserve their characteristic fluorescence for at least 6 months. Frozen sections, preserved in 10 per cent formalin, lose the yellow fluorescence characteristic of the pigment after 6 months. The various histologic structures, as is the case with other tissues, show only a whitish fluorescence.

The macrophages charged with pigment showed a luminous white fluorescence. Since the pigment was stainable with Sudan III and with fuchsin, it was obviously lipo-fuscin.

It is evident that fluorescence is a physical character of importance, which is often more sensitive, as regards both specificity and quantity, than chemical methods or even some physical methods. Barcelo, in his treatise on chemical spectroscopy, states that "spectro-analysis is a sensitive method useful in analytic chemistry, but in certain cases, there are procedures still more advantageous, for example, analysis by fluorescence."

It should be emphasized further that all the lipo-fuscins give the staining reaction of fats—(de Faría).

Discussion

In the material studied, fluorescent pigment was found in the muscular bundles, within macrophages, and in the fibrous tissues of the endometrium. The identical fluorescence of the pigment in muscle fibers and macrophages is evidence that they are indistinguishable. The pigment gives no iron reaction with potassium ferrocyanide and stains red with fuchsin and yellow with Sudan.

The quantity of pigment in the controls on vitamin E-deficient diet was the same as in animals receiving 1 mg. of progesterone or 1 mg. of alpha-tocopherol.

Conclusion

Fluorescent lipo-fuscin pigment was found in the uteri of control animals maintained on vitamin E-deficient diet, as well as in those receiving daily doses of 1 mg. of progesterone or of alpha-tocopherol. The quantity of pigment was the same in both groups. The outer layer of musculature often contained the greater quantity of pigment.

Macrophages in the intermuscular tissue between the two coats, and in the fibrous tissue of the muscle also contained fluorescent pigment.

The pigment loses its characteristic fluorescence when the sections are left in 10 per cent formalin for 6 months.

Sections mounted in polysterine dissolved in xylol conserve their fluorescence, and give excellent clear preparations.

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DEPOSITS OF FLUORESCENT PIGMENT IN THE ATROPHIC TESTES OF VITAMIN E-DEFICIENT RATS

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In 1941, Popper in a study of 22 human testes described various fluorescent substances which may be found in these organs. He noted that with the development of the testis, the vitamin A disappeared and was replaced by fluorescent yellow granules, visible in the ultraviolet, which were not present in the testes of young adults. This pigment was insoluble in alcohol and acetone.

In 1944, Sjöstrand described fluorescent pigment granules amongst the spermatogonia.

In a recent study of fluorescent pigment in atrophic human testes (Radice and Kaplan), there was described abundant yellow fluorescent pigment in the seminal epithelium. This was presumably lipo-fuscin since (a) it had the same refractivity as fats; (b) it was soluble in lipoid solvents; (c) it stained with Sudan III; (d) it was stable to ultraviolet radiation; and (e) it was demonstrable for a long time in frozen sections mounted in water or in glycerine, sealed with Balsam. Having established the presence of this pigment in atrophic human testes and knowing that it did not occur in normal testes, it was interesting to study the testes of normal and atrophic rats with this in view.

Material. Vitamin E-deficient rats were divided into four groups each comprising 4 animals:

Group I received daily 1 mg. alpha-tocopherol for 2 months.

Group II received 3 ml. of olive oil daily.

Group III received 1 mg. of Perandren daily by injection.

Group IV—controls, without supplement.

In addition, 60 rats received various doses of alpha-tocopherol acetate or wheat-germ oil, and these yielded testes ranging from normal to complete atrophy (grade 5 Mason scale).

Technical Methods. Frozen sections of testes were prepared after 48 hours' fixation in 10 per cent formalin. The sections were cut at a thickness of 25 to 50 micra. Thinner sections fell apart when placed in water, and gelatin or similar substances could not be used for embedding, since they might modify the primary fluorescence of the structures with which we were concerned.

Frozen sections were also cut from material which had been preserved for a year in 10 per cent formalin, mounted in water and examined with the ultraviolet filter (Wood's lamp). From each specimen, a block was embedded in paraffin and sections stained with hematoxylin-eosin, in order to determine the degree of atrophy, following the classification suggested by Mason.

The instruments used were a high-power ultraviolet lamp, the radiation filtered through Corning glass ultraviolet selector, and ocular protective filter type Euphos.

Atrophic testes, sectioned 24 hours after receipt of the material, showed no fluorescent substances referable to vitamin A in the seminal epithelium, thus confirming Popper's observations. On the other hand, fluorescent yellow granules were frequently encountered.

Sections from testes preserved for a year in formalin showed diffuse primary bluish fluorescence and luminosity, a phenomenon common to all tissues, which, under these conditions, lose their specific fluorescence and acquire a diffuse bluish white luminosity. Structures like muscle fibers or red blood corpuscles, which originally possess no fluorescence, become luminous after a year in formalin. The structure of the normal testis is clearly brought out. The basement membrane enveloping the seminiferous tubules often contains an abundance of whitish fluorescent material, and, in obliquely cut sections, it can be definitely shown that these fluorescent elements pertain to the basement membrane itself.

Similar structures were seen within the seminiferous tubules, which were either normal or only slightly atrophic (grade 1), or in sections where normal tubules were intermingled with atrophic ones. Fluorescent bodies were never found in the basement membrane surrounding atrophic tubules.

The germinal epithelium of the atrophied tubules, however, frequently contains fluorescent yellow refractive pigment granules. Their distribution is irregular; some tubules have an abundance of lipo-fluorescent material, others lack it completely. In general, it is found in greatest quantity in testes showing atrophy of grade 4 or 5 in the Mason scale, with the occasional exception that testes of grade 5 may lack pigment completely, and those classified as grade 1, 2, or 3 may have it. Pigment is not found in normal testes, and in atrophic testes still containing normal tubules or tubules showing only grade 1 atrophy (Mason scale), there is no fluorescent lipo-pigment. Sections treated with alcohol and xylol and mounted in polysterine dissolved in xylol show that the lipo-pigment does not disappear completely. There remain isolated formations with reddish yellow fluorescence, less luminous than those observed in frozen sections mounted in water or glycerine.

The intensity of the luminescence appears to be related to the presence of fats. The tissue fats, for example the panniculus adiposus, acquire slight diffuse luminescence when preserved for a long time in 10 per cent formalin. The atrophic germinal epithelium has a yellow fluorescence similar to that of so-called "ceroid" pigment.

Conclusions

Rat testes, rendered atrophic by avitaminosis E (grade 4 or 5 of Mason's scale) present, within the Sertoli syncytium, fluorescent pigments yellow or luminous and whitish, in frozen sections mounted in water.

Treatment with xylol dissolves the lipids in the section, leaving a smaller amount of reddish yellow pigment. The characteristic property of this pigment is its reddish yellow fluorescence; its luminescence is due to the accompanying fats.

This fluorescent lipo-pigment is not found in normal testes. Normal

testes, after a year's fixation in 10 per cent formalin, display a fluorescence in the basement membrane of the seminiferous tubules which is not present in atrophic tubules. The histologic structures of the testes, after a year's fixation, acquire a diffuse whitish fluorescence, as do other tissues.

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THE EFFECTS OF HYPO- AND HYPER-VITAMINOSIS E ON LUNG TUMOR GROWTH IN MICE*

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Introduction

Over a decade ago, several workers investigated the effects of vitamin E on tumor growth in mice and rats. Published reports of this work revealed considerable discrepancy in their results. Some workers reported that hypervitaminosis E in the mouse retarded tumor growth,^{1,2,3,4,5,6} while other investigators found that tumor growth was unaffected by vitamin E.^{7,8,9,10,11,12}

The rationale of this work was based largely on the observation of other earlier workers that vitamin E in some obscure manner played a rôle in rapid cell proliferation.^{13,14,15,16} Because of the lack of uniformity of results among previous workers, it was decided to reinvestigate this problem, using synthetic alpha-tocopherol instead of the vitamin E concentrates used by the earlier workers.

Procedure

Two hundred young strain "A" (Bar Harbor hereditary lung tumor strain) mice were used in the experiment. They were divided equally into four groups. Group 1 was maintained on a vitamin E-deficient diet. Group 2 received the deficient diet plus 2 mg. of synthetic alpha-tocopherol on alternate days. Group 3 was held on a normal stock diet. Group 4 had normal diet plus 2 mg. of alpha-tocopherol every other day. All animals received, subcutaneously, 1 mg. (1, 2, 5, 6) dibenzanthracene in $\frac{1}{4}$ cc. olive oil.

A severe diarrhea epidemic during the first month of the experiment depleted the colony to about half its original size. At the end of seven months, all of the remaining animals were autopsied. The number, size, distribution, and histologic type of the tumors were noted (TABLE 1).

Results

The incidence of lung tumors in the combined normal groups (Groups 2, 3, and 4) was 94.8 per cent. The deficient group showed an incidence of only 70.9 per cent.

Actual counts of the number of lung tumors showed that Group 2 (supplemented) had the largest number per animal, averaging 125.9. Group 4 averaged 81.0 tumors per animal; Group 3 had 71.7 per animal; and the deficient group (Group 1) had an average number of 45.6 tumors per animal.

There was a sex difference in the number of tumors of the lung. Male animals of all groups had nearly double the number of tumors per animal as the females of the corresponding group. Incidence of tumors was about the same in both sexes.

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TABLE 1
PERCENTAGE OF TUMORS IN "A" STRAIN MICE

<i>Group</i>	<i>Diet</i>	<i>Number animals autopsied</i>	<i>Lung tumors</i>	<i>Cysts</i>	<i>Subc.-tumors</i>
			%	%	%
1	E-deficient	24	70.9	75.0	33.3
2	E-deficient plus α -tocopherol	37	94.6	24.3	35.1
3	Normal	22	90.9	22.7	36.4
4	Normal plus α -tocopherol	19	100.0	36.8	47.4

On the dorsum of the shoulders at the site of the injection of the carcinogen, subcutaneous tumors appeared in some animals of all groups. They were somewhat more abundant, however, in the normal supplemented group (Group IV). In this group, 47.4 per cent of the animals had these subcutaneous tumors. In the remaining three groups, about the same incidence was noted; namely, 33.3, 35.1, and 36.4 per cent.

During the early course of the experiment, many of the animals of all groups developed a small cyst (usually about 1 cm. in diameter) at the site of the injection. The oily semi-fluid content of these cysts was suggestive of the injection media and, therefore, the question was raised whether or not the carcinogenic agent had been fully absorbed. These cysts frequently broke down and caused an ulceration of the skin which often involved a considerable area over the shoulders and forelegs. The cysts and skin lesions were more frequent in the deficient group. Therefore, the lower incidence and smaller number of tumors in the deficient group might possibly be due to poor absorption of the carcinogen and not due to the nutritional deficiency per se.

Summary

In a seven-month experiment on induced lung tumors in strain "A" mice, the following results were noted:

(1) The incidence of lung tumor was greatest in the hypervitaminosis groups.

(2) The average number of lung tumors per animal was significantly greater in the hyper-vitaminosis E groups. The deficient animals had the least number of lung tumors per animal.

(3) Male animals of all experimental groups invariably showed about twice the number of lung tumors per animal as the corresponding female of the same dietary group. No sex difference was noted in the incidence of the tumors.

(4) Subcutaneous tumors in the interscapular region at the site of injection of the carcinogen were more prevalent in the groups receiving excessive alpha-tocopherol supplementation and least prevalent in the E-deficient groups.

(5) The more frequent appearance of oil-filled cysts at the site of injection

in the deficient group suggests possibly poor absorption of the carcinogen, which may account to some extent for the low incidence of tumors in this deficient group.

(6) These findings suggest that vitamin E deficiency may reduce the incidence and number of lung tumors in Strain "A" mice as compared to the normal and hypervitaminosis E control animals.

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THE EFFECT OF CHRONIC VITAMIN E DEFICIENCY ON THE NERVOUS SYSTEM IN THE RAT.

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The problem of whether the neuromuscular disturbances seen in E-deficient rats are characterized by lesions in the nervous system, by changes in the skeletal muscles, or by a combination of both, still remains controversial. On the one hand Lipshutz,¹ Einarson and Ringsted,² Monnier,³ and de Gutiérrez-Mahoney and his associates⁴ described definite changes in various parts of the central and peripheral nervous system. On the other hand, very definite and early myopathic changes were reported by Olcott.⁵ Wolf and Pappenheimer⁶ also studied the nervous system and found no lesions there. They were inclined to attribute the conflicting observations chiefly to variations in technique resulting in different interpretations of the findings. For these reasons, we have considered it worth-while to re-investigate the pathology underlying E deficiency and have considered that there would be a greater likelihood of detecting changes in the nervous system by the use, first, of animals which resembled those employed by Einarson and Ringsted in that they had long been held on an E-deficient diet.

Material and Methods

Nine E-deficient adult rats were examined, all having been placed on the vitamin E-deficient diet⁷ at ages varying from one to twenty-one days. Of these, eight female rats were sacrificed at ages ranging from 445–534 days, after showing clinical signs of marked ataxia, muscular dystrophy, ulcerations of the skin, pigmentation, and loss of hair. Some of these animals had undergone one resorption. One male rat was sacrificed at the age of 263 days, after manifesting only mild signs of muscular dystrophy. The findings were compared with those in four control rats who had fertile matings and were clinically normal. All the animals were killed with chloroform. After removing the viscera, the cranium and vertebral canal were partially opened and the whole preparation was immersed in 10 per cent solution of formaldehyde U.S.P. for about one week before removing the cranial and spinal contents. The brachial and lumbosacral plexus and various muscles were immediately fixed in formaldehyde, Müller's and Susa's solutions. The material was then either embedded in paraffin or celloidin, or cut in frozen sections. The stains used were Pal-Weigert, Weil, Spielmeier, Bodian, Nissl (thionin), Scarlet Red, Marchi, Holzer, and Hematoxylin and Eosin.

Pathoanatomic Findings

Central Nervous System. No changes were encountered in the cerebrum, brain stem, or cerebellum. On the other hand, the spinal cord showed out-

spoken changes which were remarkably similar in all the animals. The dorsal columns were affected universally and in such a manner that, while at the lumbosacral levels the entire column was involved, the lesion, when traced through the thoracic and cervical levels, gradually became more restricted to the fasciculus gracilis, ending in the medulla at the termination of this tract in the nucleus gracilis. With myelin sheath stains, whether Pal-Weigert, Weil, or Spielmeyer, there was distinct demyelination of the dorsal funiculus, particularly of the fasciculus gracilis, which contrasted with its appearance in the normal controls. In conformity with this picture, the Bodian stain showed fragmentation and reduction of the axons in this area, within which scattered hypertrophic astrocytes were observed. A most striking change was noted in Scarlet Red preparations, consisting of abundant fat deposits in scavenger cells within the fasciculus gracilis, becoming more sparse in the fasciculus cuneatus and among the adjacent fibers coursing through the posterior horns. A similar, though less marked, change was obtained with the Marchi stain and, because of this, the latter method was later discarded. In Nissl preparations, the dorsal column appeared atrophic and retracted from the surface. By contrast with the feebly stained white matter in the control animals, the dorsal column stained deeply with thionin due to proliferation of numerous small glial nuclei, hypertrophic fibrous astrocytes, and compound granular corpuscles containing a greenish pigment. With the Holzer method, there was marked proliferation of glial fibers, filling in the otherwise unstained dorsal column. While the above changes were equally severe in the 8 E-deficient animals with the longest duration and marked clinical signs, the ninth animal, which presented only mild signs, showed a corresponding slight change but in the same location. The white matter of the rest of the spinal cord appeared normal in all the stains, and the pyramidal tract stood out particularly, as the only intact area in the dorsal column. The grey matter of the cord was everywhere well outlined and showed a normal cell content. However, some neurons within the anterior horns disclosed changes in the form of hyperchromatosis, sclerosis, vacuolization, and slight increase in fat content. These changes, while at times moderately severe, were inconstant, varying in intensity in the various E-deficient animals, and in different levels of the spinal cord of the same animal. There was no apparent correlation between these cell changes and the severity of the rest of the pathological picture or with the clinical signs. Moreover, similar sclerotic and vacuolated cells were not infrequently found in otherwise normal areas of the nervous system of the E-deficient animals, as well as in the normal controls, and were nowhere accompanied by any definite glial response.

Peripheral Nervous System. The dorsal roots showed inconspicuous changes in the form of slight demyelination, scattered fat droplets, and increase in interstitial fibers, nowhere approaching in severity the changes observed in the dorsal columns. There were no demonstrable changes in the ventral roots, spinal ganglia, and peripheral nerves.

Skeletal Muscles. Examination of any part of the muscular system disclosed signs of advanced myopathy in all the E-deficient rats, with the ex-

ception of a milder change in the one animal mentioned above. These changes consisted of diffuse atrophy of muscle fibers, increased hypolemmal nuclei, and interruption of some muscle fibers by masses of sarcoplasm surrounding large nuclei. In general, the striation of the muscle fibers was preserved and there was no evidence of fatty or fibrous replacement. No changes were noted in the muscle spindles.

Comment

The above study shows certain points of agreement, as well as certain differences, when compared with the observations of other investigators. Our findings leave no doubt that, in addition to muscular involvement, there are, at any rate, eventually, definite neural lesions in vitamin E-deficient rats. These lesions are chiefly restricted to the fibers of the dorsal columns and to some extent of the dorsal roots. In agreement with Einarson and Ringsted, the changes resemble the lesions in tabes dorsalis of man and probably account for the prominent ataxic features of E-deficient animals. With regard to the dystrophic clinical features, there is an apparent correlation with the pathological changes in the skeletal muscles, as no constant lesions could be found in either the upper or lower motor neuron systems. The pyramidal tracts were nowhere involved and the sclerosis or vacuolization of anterior horn cells was inconstant and was also encountered in the normal controls. The criticism of Wolf and Pappenheimer of technical misinterpretations may very well apply to these neuronal changes, which can be regarded as artifacts. Such a contention, however, cannot explain the lesions in the posterior columns, since, in our study, all staining methods yielded the same positive proof of the latter and contrasted sharply with the normal findings in the control animals. It may be that the discrepancy in some of the findings of various authors can be attributed to differences in duration, as well as severity, of the dietary deficiency. In Wolf and Pappenheimer's series, the animals were 6 to 12 months of age, while those studied by Einarson and Ringsted were approximately 12 to 24 months old and those studied by Monnier 14 to 23 months. De Gutiérrez-Mahoney and his associates studied second generation animals, 12 months of age. These would be expected to show more severe changes than first generation animals of the corresponding age. In our series, the 8 animals showing advanced changes were 15 to 18 months old, whereas the only animal with minimal pathology was approximately 8 months of age. Whether differences in diets employed by various investigators play a rôle remains undetermined.

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STUDIES ON THE HISTOPATHOLOGY OF VITAMIN E DEFICIENCY

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The histopathology of vitamin E deficiency has already given rise to numerous contributions, some of which have become classic. We have undertaken this study ourselves not so much to confirm any findings as to elucidate some points which are still debated.

The chronaximetric test, which has been used by Lecoq *et al.*,¹ has allowed us to emphasize the precocity and importance of neuromuscular disturbances in vitamin E deficiency which depend on a state of encephalo-medullary excitation. These signs, which appear from the 25th to the 35th day, precede the clinical manifestations and sexual disturbances. The question was whether they accompany more or less rapidly the lesions in the central nervous system which have been observed by Einarson and Ringsted,² as well as by Monnier,³ but denied by Wolf and Pappenheimer.⁴

We used white rats 40–50 grams in weight and submitted them for 8 or 9 months to the regimen BR 28 of Evans, after which they were sacrificed. These rats continued to grow during the first 4 months, after which their weight remained stationary. They were, thus, smaller than normal (120 to 160 grams) and had rough fur and exophthalmia. When males and females were combined in cages they did not reproduce.

Previously, lesions in the nervous system had been observed in rats which had been subjected to prolonged vitamin E deficiency for 10 or 16 months and had shown marked symptoms in which, we believe, cachexia could have been an added factor. This is the reason which made us interfere at the 8th or 9th month, when there was already a slight loss of weight in some of the subjects. Organs were removed immediately after the sacrifice of the animals. Fixation and staining were carried out by the usual procedures.

Our attention was first directed to the genital organs. In the male, our observations agree with what is already known. There are no modifications of the excretory segment. However, one can observe, in a certain number of seminiferous tubules, the persistence of some spermatogenesis (spermatozoa immobilized or agglutinated as already described) without ever finding any spermatozoa in the ducts of the epididymis. Leydig cells show hyperplasia. The prostate is essentially normal. In the female, the uterus does not seem to show any change. In the ovaries, the stroma and the vascularization are normal, and the interstitial gland is very much developed. No *corpus luteum* can be detected. However, developmental anomalies of the Graafian follicle (which is never seen to have reached maturity) make us think that there is an actual disturbance in follicular development and not an absence of progestational transformation of the follicles.

The myocardium shows slight irregularity in the staining affinity of the muscle fibres, some of which seem very pale and show the beginning of cloudy swelling. There are no modifications in the striations or in the position of

the nuclei. These alterations justify the use of vitamin E in the treatment of certain cardiac conditions.

In the bone marrow, a marked increase in the number of megakaryocytes is observed; the other elements seem to be in normal proportions. The lymph nodes show myeloblasts and myelocytes in the blood vessels of the medulla. Finally, a hyperplasia is observed in the white substance of the spleen, caused by increased volume of the Malpighian corpuscles and the presence of numerous early forms of granulocytes in the venous sinuses. This truly shows a defense reaction against anemia.

In the endocrine glands, the thyroid seems to be particularly affected. The irregularity in the size of follicles and the variation in the staining of the colloid, as well as the cuboid or flat epithelium, suggest a hypofunctional state of the glands. We have not observed any changes in the anterior or posterior hypophysis. It seems that, for this gland, the disturbance is functional rather than on the basis of a lesion.

We were interested in seeing whether the effect on the nervous centers was the same. The chronaximetric disturbances, which are manifested very precociously and persist during the whole development of vitamin E deficiency, point to the existence of a nervous disturbance of a polyneuritic type. We have not, however, observed any change either in the brain and spinal cord or in the ganglia and peripheral nerves. The anterior horns of the spinal cord show cells which are cytologically normal. The dorsal roots and the posterior tracts are normal, as are, also, the bundles of Goll and Burdach. The nerve trunks themselves are intact.

No alterations are found in the cerebral cortex or the bulb, but in the cerebellum there are numerous localized foci of degeneration in the area of the white substance, with vascular congestion and hemorrhages. This confirms the observations of Adamstone⁵ in chicks.

The skeletal muscles show irregular staining of the striated fibres, with areas of edema. There are no changes in the position of the nuclei.

Conclusions. It appears that vitamin E deficiency first results in the acidotic changes of metabolism which are really responsible for the neuromuscular disturbances.

It is difficult to say whether the metabolic disturbances lead to the endocrine changes or whether the latter give rise to the humoral modifications. This latter appears to be more probable. Endocrine dysfunctions are not necessarily accompanied by lesions.

Our observations, which were made before the effect of cachexia was superimposed on the vitamin E deficiency, show that the morphological muscle changes are still minimal, while the changes in the cerebellum are already marked. This leads us to believe that the effect on the cerebellum conditions the process of muscular degeneration. On the contrary, one does not observe (in agreement with Wolf and Pappenheimer) any injury to the spinal cord or to the cerebrum.

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ACTIONS OF VITAMIN E ON THE NON-DEFICIENT ORGANISM

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Using the measurement of chronaxie (a very sensitive test which allows the detection of latent disturbances in nervous functions), we have demonstrated¹ that vitamin E deficiency in the rat begins to manifest itself from the 25th day of deficiency by encephalomedullary excitation, which leads to later muscular disturbance about the 35th day. We have also shown² that in the pigeon the same signs appear more precociously (from the 10th to the 12th day). Thus, vitamin E deficiency takes its place, with the avitaminoses A, F, and K, in the group of fat-soluble acidotic deficiencies. This is confirmed by the transitory curative effect of superimposed alkalotic deficiency such as rickets.^{3, 4} To complete this work, we set out to study the effect of vitamin E (in doses of several milligrams) on the normal organism, without any deficiency.

Chauchard has already shown⁵ that a parenteral injection of such a dose of vitamin E, in the form of alpha-tocopherol in oil solution, results in diphasic oscillations of the nerve chronaxie (excitation, followed by the inhibition of the encephalomedullary centers) and has concluded that the therapeutic effect of this vitamin, as well as that of other vitamins, is not necessarily dependent on a state of latent deficiency, but could, in some cases, depend only on its pharmacodynamic activity. If such injections are given daily to the rat or the guinea pig, a state of latent hypervitaminosis is achieved, which consists in a permanent increase of the nerve chronaxie.

An animal prepared in such a manner can be used to study the synergistic or antagonistic effect of vitamin E. If it is subjected⁶ to injections of ammonium chloride, which produces acidosis, its chronaxie returns to normal, while the same does not occur with alkalizing injections of sodium bicarbonate. Vitamin E, like vitamin A, seems to have an alkalizing effect on the normal organism, which bears out the acidotic nature of the deficiency which it compensates. Besides, intravenous injection of vitamin E raises the alkaline reserve of plasma.⁷ According to these observations, vitamin E is antagonistic to the acidotic vitamins (C, D, and choline), while its effects are not suppressed by the alkalotic vitamins (B complex, vitamin A, and adrenochrome).

Regarding the chemical mediators, we have observed that adrenaline inhibits the chronic nervous changes of vitamin E (which shows the antagonism of these two substances), while neither acetylcholine nor histamine oppose the action of vitamin E.

Utilizing the same technique, we have studied the effect of several hormones or endocrine extracts. Follicular fluid, anterior pituitary extract, and thyroxin are antagonistic to vitamin E, while progesterone and posterior pituitary extract have no effect. It is interesting to consider these results in parallel with the anterior pituitary and thyroid hypoplasia of vitamin E deficiency, with the equilibrium of vitamin E and estrogen in the blood, and with the progesterone insufficiency in the subject with vitamin E deficiency.

The measurement of the chronaxie also allows us to test variations in the

excitability of visceral muscles *in situ*. In avitaminosis E there is an increase of all the visceral chronaxies, which correlates with acidosis.⁸ But, while the figures are only doubled for the intestine, the action is much more selective (which is not true of other avitaminoses) on the genital organs, the chronaxie of the uterus changing from 0.5 to 25 milliseconds and that of the seminal vesicle from 2.5 to 45 milliseconds.

Thus, the uterus shows a variation in chronaxie comparable to that caused by progesterone, anterior pituitary extract, or castration, and opposite to that caused by estrogen. In the case of the seminal vesicle, the effect is parallel to that of testosterone.

The application of vitamin E to the uterus of a normal animal prolongs its chronaxie, producing an effect similar to that of a state of deficiency, which is often the case with vitamins. In agreement with this variation, the action of estrogen (which diminishes the chronaxie of the uterus) is decreased, while that of the anterior pituitary extract (which increases the chronaxie) is increased.

Conclusions. Like other vitamins, vitamin E (alpha-tocopherol), outside of the state of deficiency, seems to have a pharmacodynamic effect. We have emphasized, in particular, its pronounced and chronic effect on the encephalomedullary centers.

While avitaminosis E is classified among the deficiencies producing an acidosis, vitamin E appears to have alkalizing properties.

As an antagonist of the acidotic vitamins and of adrenalin, vitamin E resembles vitamin A, and one can conceive that their association has something to do with action against arterial hypertension. It does not show, by contrast, any of the characteristics of the anti-allergic vitamins (D, C, P) which are antagonistic to histamine and acetylcholine.

The antagonism and the synergistic effects with the endocrines, which we have observed, are to be added to the few facts which are known about relationships between hormones and vitamin E.

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THE EFFECT OF TOCOPHEROLS, OLIVE OIL, TESTOSTERONE, AND CORPUS LUTEUM EXTRACT (LUTEOCICLINA) UPON THE LESIONS CAUSED BY VITAMIN E DEFICIENCY

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The present study summarizes the more interesting modifications noted in vitamin E-deficient rats, produced by the administration of tocopherols and endocrine products such as testosterone in the male and of corpus luteum extract in the female. It also summarizes the effect of dietary additions of olive oil, which as shown in a previous paper, is devoid of vitamin E.

The rats were killed by decapitation. The macroscopic study included a complete autopsy. The principal organs were weighed and examined for fluorescence with the Wood lamp. Pieces of the tissues were fixed in 10 per cent formalin. Frozen sections were mounted in water and examined unstained for histophysical study (fluorescent microscopy). Other sections were stained with Sudan III and hematoxylin for demonstration of lipids or Sudanophilic material. Part of the tissues were embedded in paraffin and sections stained with hematoxylin-eosin and by the Gallego method for the demonstration of lipo-fuscin. Some sections were used for the microchemical demonstration of iron by the methods of Tirmann and Schmelzer or the Schmieden reaction.

Macroscopic Appearance. Vitamin E-deficient rats, exposed to ultraviolet radiation of wave length 3650 Å (Wood lamp), present a milky whitish fluorescence of the panniculus adiposus, which is considerably reduced in amount and, in normal animals, is nonfluorescent. This phenomenon is particularly striking in the abdominal tissue fat, the consistency of which is reduced as compared with that of normal animals. The panniculus adiposus of normal rats presents, as is known, a brightly luminous yellow fluorescence. These observations on fluorescence confirm those recorded in a preceding publication.

The panniculus adiposus of rats on a vitamin E-deficient diet is reduced in amount, but is augmented by administration of vitamin E. Vitamin E-deficient animals receiving a supplement of olive oil daily have less panniculus adiposus than normal rats. The organs grossly present a dull violet color, which one may call negative fluorescence (failure to fluoresce), whereas those of normal animals have slight greenish-yellow fluorescence.

The uterus of vitamin E-deficient rats has an intense chestnut brown color, and the uterine horns exposed to long ultraviolet waves (Wood lamp) exhibit striking yellow-brown fluorescence of moderate luminous intensity. This was slightly reduced in the rats which, after a period of prolonged deficiency were given alpha-tocopherol supplement, but, as will be shown, there is no apparent reduction in the amount of pigment in the histologic sections. It would be necessary to continue the tocopherol administration for a longer period in order to draw a positive conclusion on this point.

TABLE 1

<i>Material studied</i>				
Rat No.	1053	—	Avitaminosis E	2 mg. Progesterone inj. daily
" "	1054	" "	" "	" " " " " "
" "	1055	" "	" "	2 mg. alpha-tocopherol <i>per os</i> daily
" "	1056	" "	" "	Control
" "	1057	" "	" "	" "
" "	1052	" "	" "	2 mg. alpha-tocopherol <i>per os</i> daily
" "	1051	" "	" "	" " " " " " " "
" "	1050	" "	" "	" " " " " " " "
" "	1049	" "	" "	Control
" "	1048	" "	" "	" "
" "	1047	" "	" "	" "
" "	1046	" "	" "	3 ml. olive oil <i>per os</i> daily
" "	1045	" "	" "	" " " " " " " "
" "	1044	" "	" "	" " " " " " " "
" "	1043	" "	" "	1 mg. Perandren by inj. daily
" "	1040	" "	" "	" " " " " " " "
" "	1042	" "	" "	" " " " " " " "
Rats of Litter 969				
No.	391	" "	" "	3 mg. olive oil <i>per os</i> daily
" "	388	" "	" "	1 mg. alpha-tocopherol "
" "	393	" "	" "	1 mg. Perandren by inj. daily
" "	394	" "	" "	Control

Rats made E-deficient and then injected with 1 mg. of testosterone daily, showed testicular atrophy of grade 5 (Mason scale), the germinal epithelium being replaced by Sertoli syncytium. There was marked hypertrophy of seminal vesicles and prostate, however, as shown in FIGURE 1. In the upper part of the photograph are shown the seminal vesicles and prostates of 3 control rats on E-deficient diet throughout the experiment. In the lowest bracket, may be seen the seminal vesicles and prostates of rats receiving a daily supplement of 2 mg. of alpha-tocopherol. The size and structure of these organs is identical in the two groups. In the middle bracket are shown seminal vesicles and prostates of E-deficient rats which had been given 1 mg. of Perandren daily. The increase in size of these structures is obvious, and can easily be estimated from the metric scale in the right of the photograph.

Microscopic Findings. Vitamin E-deficient rats, as well as those subsequently given alpha-tocopherol, have in the uteri an abundance of yellow-golden pigment with a chestnut tinge, characteristic of the lipo-fuscin described by Barrie, Martin, Moore, Sweeten, Mason, Dutra, and de Faría in avitaminosis E rats and by Popper, György, and Goldblatt in other lesions.

This pigment occurs in both muscular layers, as well as within macrophages between the muscle bundles (Dutra and de Faría). The macrophages are large cells with a centrally placed nucleus. Their cytoplasm is filled with numerous round pigment granules of uniform size. The cells are often in the vicinity of blood vessels of medium caliber.

The pigment is strongly fuchsinophilic when stained by Gallego's method and takes an orange color with Sudan III. It gives no Prussian blue reaction. It is fluorescent at 3650 Å, when examined with the ultrared Corn-



FIGURE 1. Top row: seminal vesicles and prostates of 3 vitamin E-deficient rats.
Lowest row: the same organs of 3 vitamin E-deficient rats which had received 2 mg. daily of alpha-tocopherol.
Middle row: the same organs from 3 vitamin E-deficient rats treated with one mg. Perandren daily.

ing glass selector and a protective filter (Type “Euphos”) in the ocular of the microscope.

Sections mounted in a solution of polysterine in xylol gave the best definition and preserved the physical character of the pigment for a year. In frozen sections preserved in formalin, the characteristic fluorescence of the pigment gradually disappeared, and in a few months all structures showed only a whitish luminosity.

The number of macrophages and the quantity of pigment were similar in

control rats and those given daily injections of one mg. of testosterone, or those receiving one mg. of alpha-tocopherol. These observations confirm those of Sweeten, who states that the uterine pigmentation of E-deficient rats is not cured by administration of vitamin E.

The rats which received one mg. of Perandren daily had testicular atrophy (4 or 5 on Mason's scale) comparable to that of the controls without supplement. The germinal epithelium was entirely replaced by an oedematous syncytium which, in places, contained fluorescent pigment, representing lipoids stainable with Sudan III and giving pronounced brilliant yellow-white luminescence. After treating the sections with lipid solvents, a slightly luminous fluorescent reddish-yellow pigment remained. The typical feature of the pigment is its yellow fluorescence. The increase in luminosity is due to the aggregation of fatty substance which shows, after prolonged sojourn in formalin, the whitish fluorescence common to most organic tissues.

Vitamin E-deficient rats show a reduction in the amount of Vitamin A detectable by its yellow-green fluorescence in frozen sections of liver or adrenal. Vitamin E-deficient rats which had received Perandren show a slight increase in the amount of vitamin A in the organs in which it is normally stored. In frozen sections of liver stained with Sudan III, there are always found small amounts of lipoids within the hepatic cells. In some animals it is more abundant. It is difficult to correlate the amount with the various supplements administered. The small variations noted may be ascribed to individual variation, such as is ordinarily encountered in experiments of this sort. In every case, we believe that the amount of hepatic lipoid is less than in the normal animal. Rats which had received 3 ml. of olive oil did not show any increase in stainable liver fat.

Conclusions

(1) Male rats on a vitamin E-deficient diet, when given testosterone, develop marked hypertrophy of seminal vesicles and prostate, but the testis is atrophic (grade 5 of Mason's scale).

(2) Rats given olive oil show no increase in hepatic fat, and the amount of A, indicated by its greenish fluorescence, is comparable to that of the controls on unsupplemented vitamin E-deficient diet.

(3) The uteri of E-deficient rats show intense fluorescence, grossly less marked in those given vitamin E therapy. Microscopically, however, the pigment content is the same in both groups.

(4) The fluorescent lipo-fuscin pigment in the uteri of vitamin E-deficient rats is not modified by Progestine or alpha-tocopherol.

(5) The livers of vitamin E-deficient rats contain a diminished amount of vitamin A, as shown by fluorescent microscopy of frozen sections mounted in water.

II

TOCOPHEROLS AND THEIR ESTERS IN ENZYME AND TISSUE FUNCTIONS: INTRODUCTORY REMARKS

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The previous papers provided a suitable setting for the consideration of enzyme and tissue functions of tocopherol. Those papers also reminded us that vitamin E was accepted as a new member of the family of vitamins because of the morphological and functional alterations produced by its absence. Such observations have been extended and multiplied. Perhaps no other of the vitamins mysteriously affects so many and so varied body processes. Many years ago Sir Frederick Hopkins described the goal of biological chemistry in these words: "biochemical and physiological activities will in the end reach to a description of living systems which, in so far as they are chemical systems, will be complete." Following the brilliant discovery of the chemistry of tocopherol, the quest for the mechanism of its action began. This quest is continuing with ever-increasing vigor and with ever-widening horizons.

An early guide-post in this search was the ability of tocopherols to delay the autoxidation of unsaturated fats. For those who followed this clue, antioxidants, synergists, and co-vitamin became words to conjure with. Some interesting matters relating to peroxides and abnormal pigments have come to light, as we have already learned. Another guide-post was the enhanced consumption of oxygen by the muscles of certain species when they lack vitamin E. Those who followed this gleam have been able to make out a few land-marks, but, like objects seen in a fog, the outlines are dim, so dim that their reality may sometimes be doubted.

The biochemists of my generation look with admiration and confidence to our younger colleagues who have become expert in the subject of biological oxidation. As they continue to wrestle with the problems posed by vitamin E, their efforts will ultimately be rewarded here as they have been elsewhere. The papers which follow will give a measure of their progress.

EFFECTS OF THE TOCOPHEROLS AND THEIR PHOSPHATES ON ENZYME SYSTEMS*

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Vitamin E, or more specifically α -, β -, γ -, and δ -tocopherols, functions in the body in some manner which is still unknown. The current working hypothesis is that vitamin E acts primarily and specifically through some enzyme system and, secondarily, in a non-specific manner as a physiological antioxidant. The general methods for approaching this problem fall logically into three main divisions: first, are the studies of the changes in enzyme systems which result from vitamin E deficiency; second, and very closely allied, are studies of changes in enzyme systems in which the tocopherols have been added *in vivo* and *in vitro*; and, third, are studies dealing with the quite separate consideration of the action of the tocopheryl esters *in vivo* and on isolated enzyme systems.

Severe vitamin E deficiency leads to a form of muscular dystrophy in many animals, characterized pathologically by the gradual replacement of normal muscle cells with fibrous tissue. Dystrophic tissues have an increased oxygen consumption¹ which may result in a high rate of respiration for the whole animal. A deficiency of vitamin E is unique in that it results in a stimulation of respiratory mechanisms. In addition, the dystrophy syndrome is accompanied by alterations in chemical composition and in functional behavior, of which the most striking are decreased muscle creatine² and marked creatinuria. The succinic dehydrogenase system is apparently unaffected,³ but changes have been noted in the response of the lactic dehydrogenase system to digitoxin.⁴ General disturbances in phosphorylation mechanisms have been noted in dystrophic muscle, including the depression of coupled phosphorylations of creatine and the diminution of adenosine triphosphatase.⁵ The decrease in cholinesterase content of the tissues in vitamin E deficiency⁶ implies a close association of vitamin E and acetyl choline synthesis.

The administration of α -tocopherol results in an immediate decrease in or even complete remission of the various changes associated with muscular dystrophy. When administered to normal animals, it has been reported to modify the metabolism of lipids and phospholipids,⁷ to enhance phosphorylations,⁸ and to improve the metabolism of carbohydrates.⁹ The addition of α -tocopherol, even when solubilized, to *in vitro* systems has no effect in most cases. However, α -tocopherol in minute concentrations has been shown to stimulate acetylcholine synthesis.¹⁰ Furthermore, it inhibits lipoxidase, probably due to its action as an antioxidant.¹¹

The study of the action of the tocopheryl esters on a number of isolated enzyme systems is currently of great interest. α -Tocopheryl phosphate (α -TPh), because of its water solubility, has played a commanding rôle. Orally administered, α -TPh can be readily hydrolyzed in the body,¹² and

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many of its *in vivo* reactions parallel those resulting from the administration of unesterified tocopherols. α -TPh, added *in vitro*, has been shown markedly to inhibit practically every enzyme on which it has been tested. The inhibition of the succinic oxidase system apparently involves both a specific action¹³ and a non-specific secondary mechanism involving calcium removal¹⁴ and subsequent inhibition by oxalacetate. Diphosphopyridinenucleotidase is likewise strongly inhibited by α -TPh.¹⁵ α -TPh has been reported to have no effect on coupled phosphorylations⁵ but to stimulate phosphocreatine synthesis under certain conditions.¹⁶ α -TPh inhibits a number of other enzymes, including liver acid phosphatase,¹⁷ fatty acid oxidase,¹⁸ trypsin, and other proteases, and it is antithrombic.¹⁹

Since previous reports indicated that α -TPh inhibited the succinic oxidase system, investigation of the action of the other tocopheryl phosphates was undertaken to determine if they functioned in a similar fashion. γ -TPh and δ -TPh were added to the succinic oxidase system as previously described for α -TPh.¹⁴ β -TPh has not yet been prepared.

The results of these experiments, as shown in TABLE 1, indicate that α -,

TABLE 1
INHIBITION OF THE SUCCINIC OXIDASE SYSTEM BY α -, γ -, AND δ -TOCOPHERYL PHOSPHATES

Compound	Tocopheryl phosphate concentration at 50% inhibition, molar $\times 10^4$	
	Ca Conc. $4 \times 10^{-4}M$	Ca Conc. $8 \times 10^{-4}M$
α -Tocopheryl phosphate	4.3	7.7
γ -Tocopheryl phosphate	3.2	4.8
δ -Tocopheryl phosphate	4.9	7.2

The succinic oxidase assay method of Schneider and Potter²¹ was used. Aqueous solutions of the tocopheryl phosphates were added before the calcium chloride solution. The α - and γ -tocopheryl phosphates were Ca. 95 per cent pure and the δ -tocopheryl phosphate represents a mixture of 60 per cent δ - and 40 per cent γ -tocopheryl phosphate.

γ -, and δ -TPh inhibit the succinate oxidase system to approximately the same degree. Since α -, γ -, and δ -tocopherols differ widely in biological potency it would appear that the inhibitive properties of the tocopheryl esters bear no relationship to the biological function of vitamin E. The addition of twice the normal amount of calcium chloride relieved the inhibition in all three esters to approximately the same extent. These results indicate that the other tocopheryl phosphates inhibit this system by a mechanism involving the calcium effect which has been postulated previously for the α -TPh system.¹⁴ The tocopheryl phosphates probably do directly inhibit the succinate oxidase system, but a considerable portion of the observed inhibition is attributable to their indirect action on calcium concentration. Any assay method which includes calcium, magnesium, or other alkaline earth metal as part of the reaction mixture is, therefore, not suitable for the determination of the effect of the addition of the tocopheryl phosphates.

Since α -TPh inhibits such a varied group of enzymes, and since α -, γ -, and δ -TPh inhibit to the same degree, it appears that the tocopheryl phosphates

are functioning as non-specific protein inhibitors. An investigation was undertaken to determine if a non-specific combination with proteins could be demonstrated by viscometric methods. A buffered solution of crystalline bovine plasma albumin was prepared, and viscosities were determined at several dilutions. α -TPh was added to similar solutions and viscosities again determined at several dilutions. Viscosities of the protein solutions were determined by an Ostwald viscometer in the usual manner. Corrections for the kinetic energy was made in determining the relative viscosities. The *inherent viscosities* (\ln relative viscosity/concentration at finite concentrations) were computed for each solution and plotted as shown in FIGURE 1. The *intrinsic viscosity* is obtained by extrapolation of inherent

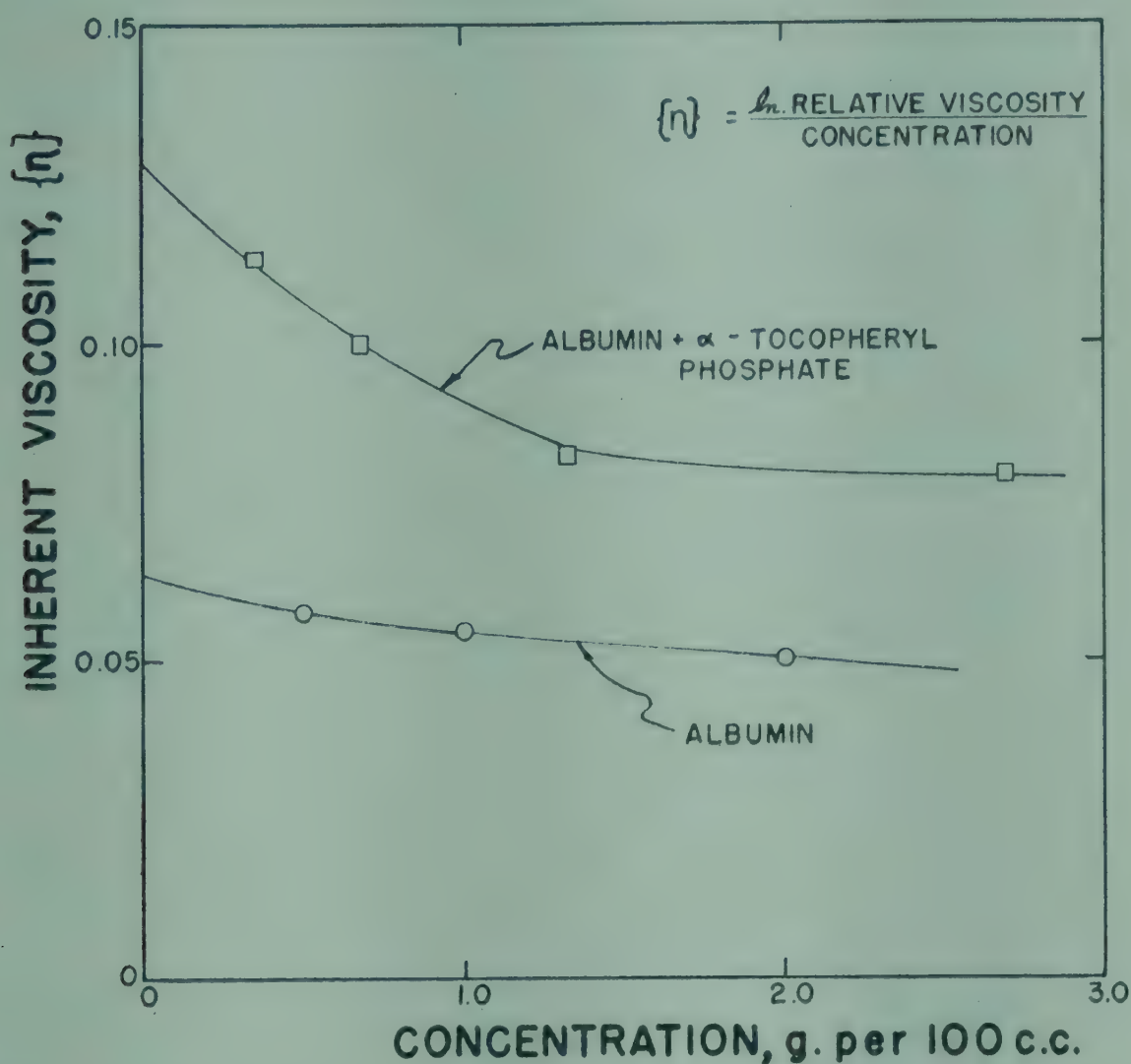


FIGURE 1. Effect of d, α -tocopheryl phosphate on the viscosity of bovine plasma albumin solutions. A 2 per cent aqueous solution of Armour's crystalline bovine plasma albumin in 0.02 M phosphate buffer pH 8.0 was used as the stock solution from which dilutions were made. When d, α -tocopheryl phosphate was present, it constituted 0.7 per cent of the stock solution. The weight ratio between d, α -tocopheryl phosphate and protein was 0.35 to 1.0.

viscosity data at several concentrations to infinite dilution. The intrinsic viscosity is a measure of the asymmetry of the molecule and is independent of concentration.

It is evident that the addition of α -TPh resulted in substantial changes both in the inherent viscosities at similar concentrations and in the intrinsic viscosity. This indicates that the addition of α -TPh to a protein solution results in the formation of a more asymmetric molecule with an increased viscosity. Combination of protein and α -TPh is the simplest explanation for the increased intrinsic viscosity. Since it has been shown that α -TPh will combine with plasma albumin, it is reasonable to conclude that it might

combine in a similar fashion with an active enzyme and probably block the active centers.

Considerable confusion exists with respect to the comparison of the actions of α -tocopherol and its phosphate. The latter is not known to exist in the body, and active hydrolytic mechanisms¹² are probably adequate to release free tocopherol. α -TPh is not an antioxidant, possesses no oxidation reduction potential, and is oxidized with difficulty. Frequent direct comparisons are made between *in vitro* concentrations of α -TPh and *in vivo* levels of tocopherols. Such comparisons are generally unwarranted. The *in vitro* effects of α -TPh probably bear no relationship to the biological functions of vitamin E.

Since numerous difficulties of interpretation exist when tocopheryl esters are used in enzyme systems, it would seem reasonable to turn to methods by which α -tocopherol itself can be added to *in vitro* systems. It is impossible to add free tocopherol to aqueous systems because of its insolubility. A number of attempts have been made to solve this problem by the use of chemical solubilizers without achieving satisfactory results. The use of desoxycholic acid has been described with negative results.²⁰ The Tweens can be used to form aqueous dispersions of the tocopherols, but these solutions have a deleterious effect on many enzymes. None of these procedures simulates the physiological transport mechanisms of tocopherol in the body.

Two procedures have been developed by which relatively large quantities of α -tocopherol can be dissolved in blood plasma or protein solutions. One method consists of slowly adding a concentrated solution of α -tocopherol in dioxane to the protein solution with rapid stirring. The resulting solution may be somewhat turbid but can be clarified by high-speed centrifugation. The preferable procedure is to homogenize a mixture of the aqueous solution of the protein and α -tocopherol in a Potter-Elvehjem glass homogenizer and follow by centrifugation. The latter method avoids the difficulties resulting from dioxane in the solution. With a 2 per cent protein solution, an α -tocopherol concentration in the supernatant of 2 to 3 milligrams per ml. can be readily achieved as a stable, slightly opalescent solution. The α -tocopherol present constitutes 1–2 per cent of the weight of total protein. The α -tocopherol-plasma complex was fractionated with either ammonium sulphate or ethanol at low temperatures, and the resulting fractions all contained α -tocopherol. A large number of amino acids, partially hydrolyzed proteins, and native proteins have been tested for their ability to form conjugates with α -tocopherol, and only native proteins have been found satisfactory. Examples of these are: blood plasma, blood plasma fractions, reconstituted serum, egg albumin, and crystalline bovine plasma albumin. Some of these proteins were lipid-free and the conjugate therefore appears to involve a tocopherol-protein linkage without the mediation of lipids. Thus, a technique has been developed by which relatively large quantities of α -tocopherol can be introduced into aqueous enzyme systems in a nearly physiological manner.

Additional data bearing on the formation of an α -tocopherol-protein complex were obtained by viscometric methods as outlined previously for α -TPh.

The viscosities of solutions of crystalline bovine plasma albumin were compared with those obtained from α -tocopherol-albumin conjugates as prepared by the homogenization procedure. The results in FIGURE 2 show that

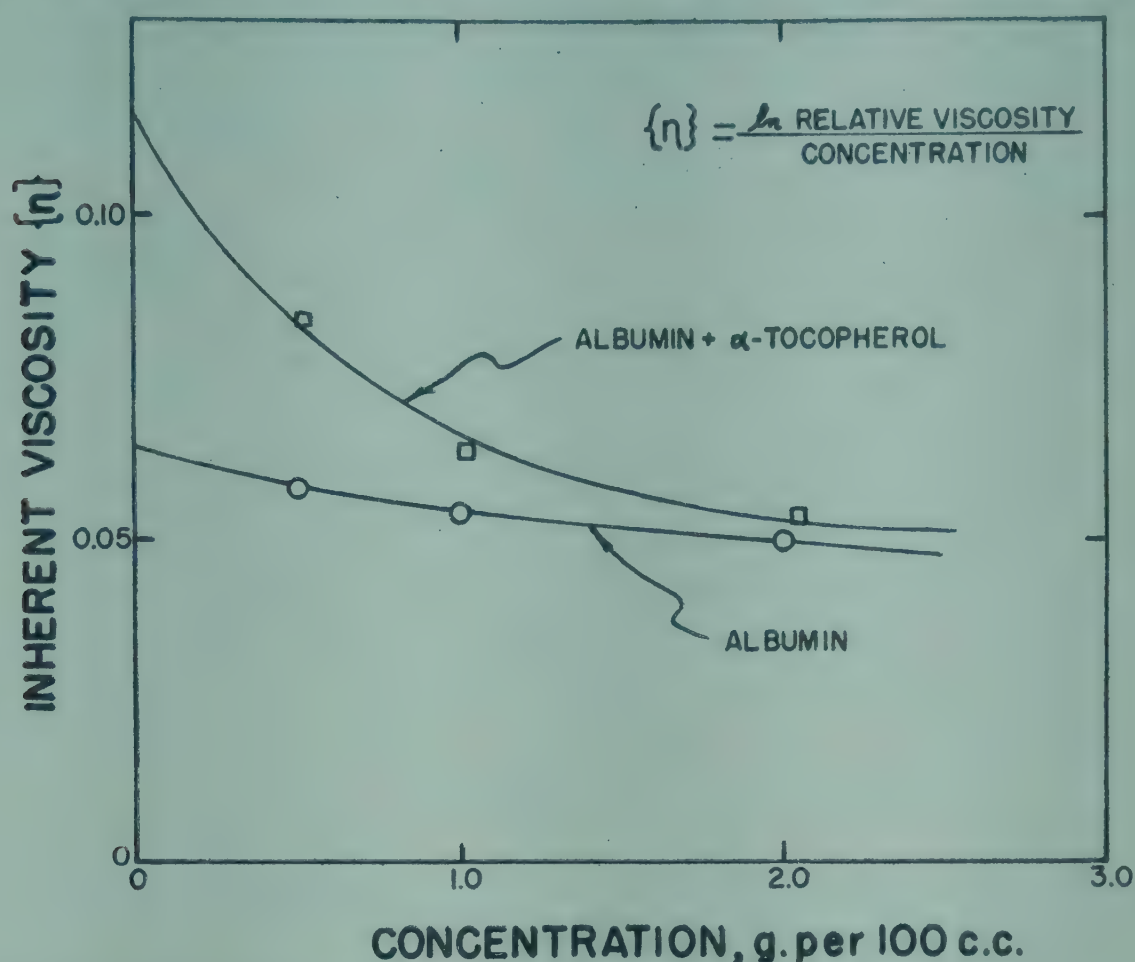


FIGURE 2. Effect of d, α -tocopherol on the viscosity of bovine plasma albumin solutions. A 2 per cent aqueous solution of Armour's crystalline bovine plasma albumin in 0.02 M phosphate buffer pH 8.0 was used as the stock solution from which dilutions were made. d, α -Tocopherol was added to the albumin solution and the mixture homogenized. Following centrifugation, the supernatant, which contained 0.54 mg. of d, α -tocopherol per ml. of protein solution, was used as the stock solution. The weight ratio between d, α -tocopherol and protein was 0.027 to 1.0.

the addition of α -tocopherol to the protein solution resulted in a significant change in the intrinsic viscosity. These observations indicate that a complex of α -tocopherol and protein is formed, since the contribution of the albumin molecule to the viscosity of the solution has been substantially changed.

The effect of the α -tocopherol-albumin conjugate on the succinic oxidase system was determined by adding this conjugate to the system. Results in TABLE 2 show that under normal conditions the succinic oxidase system

TABLE 2
EFFECT OF α -TOCOPHEROL-ALBUMIN CONJUGATE ON THE SUCCINIC OXIDASE SYSTEM

Experiment	Q_{O_2} (% of maximum rate of control)		
	1st hr. (max.)	2nd hr. (av.)	3rd hr. (av.)
Control	100	80	71
" + Albumin	94	79	72
" + α -Tocopherol-Albumin Conjugate	75	79	72

Twenty mgs. and 1 mg. of bovine plasma albumin and d, α -tocopherol respectively were added per reaction vessel. A sample of pure d, α -tocopherol was weighed into a homogenizer tube, the protein solution added, and the mixture thoroughly homogenized. The mixture was not centrifuged. The maximum rate of the control was measured over a 30-minute period, after which progressive inactivation was observed.

achieves its maximum rate during the first hour and, during the second and third hour, this rate is substantially decreased. The addition of albumin to the solution has no effect upon the rate at which the enzyme loses activity. On the addition of α -tocopherol-protein conjugate, an inhibition of the enzyme was observed amounting to approximately 25 per cent. However, the maximum activity was substantially maintained over a three-hour period, indicating a protection of the enzyme against inactivation not observed in the control sample.

A second experiment was performed in which α -tocopherol was homogenized with the enzyme preparation before addition to the succinic oxidase system. It will be noted from the results in TABLE 3 that the control sample

TABLE 3
EFFECT OF α -TOCOPHEROL ON THE SUCCINIC OXIDASE SYSTEM

Experiment	α -Toc. mg./ml.	Q_{O_2} (% of maximum rate of control)		
		1st hr. (max.)	2nd hr. (av.)	3rd hr. (av.)
Control	—	100	87	70
"	+ 1.0	102	98	80
"	+ 2.0	71	71	68

Aliquots of the tissue homogenate used in the control runs were rehomogenized with pure d, α -tocopherol, adding either 1 or 2 mgs. of d, α -tocopherol per ml. of tissue homogenate. Reaction vessels contained either 0, 0.5, or 1.0 mgs. of d, α -tocopherol.

reached its maximum rate during the first hour and then substantially lost activity during the second and third hours. The addition of α -tocopherol to the extent of 1 milligram per ml. resulted in the maximum rate being maintained during the first two hours and a slower rate of inactivation during the third hour. The addition of two milligrams per ml. of α -tocopherol resulted in some denaturation of the enzyme preparation itself, but the rate of loss of activity over the three-hour period was negligible.

The inactivation of enzyme preparations containing sulfhydryl groups as a portion of the active centers, such as succinic dehydrogenase, results primarily from oxidation of the sulfhydryl groups. When α -tocopherol was either added as an α -tocopherol-albumin conjugate or homogenized directly to form an α -tocopherol-liver protein conjugate, substantial preservation of the original activity of the system was achieved. This stabilization is probably due to α -tocopherol acting in its postulated rôle as a physiological antioxidant.

Summary

(1) A brief discussion is presented of the effects of the tocopherols and their phosphates on enzyme systems.

(2) The phosphates of α -, γ -, and δ -tocopherol inhibit the succinic oxidase system to approximately the same degree. This inhibition is relieved in every case to the same extent on the addition of supplementary calcium ions.

(3) α -Tocopheryl phosphate is indicated to be a non-specific inhibitor. Viscosity studies show that it can form a complex with bovine plasma albumin. The *in vitro* effects of the tocopheryl phosphates probably bear no relationship to the biological function of vitamin E.

(4) A method is presented for the formation of α -tocopherol-protein conjugates which resemble those found in physiological transport systems.

(5) In the presence of this α -tocopherol-protein conjugate, the succinic oxidase system is protected against inactivation for a period of approximately three hours, probably due to the action of the α -tocopherol as a physiological antioxidant.

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Discussion of the Paper

K. L. ZIERLER (*The Johns Hopkins University and Hospital, Baltimore, Maryland*): Transfer of alpha-tocopheryl phosphate action *in vitro* to an *in vivo* system is not possible. The question of its detergent-like properties *in vitro* has assumed major importance.

S. R. AMES: Regarding the "calcium effect," this mechanism of tocopheryl phosphate inhibition is probably only of secondary importance *in vivo*, but it contributes markedly to the observed inhibition in the succinic oxidase assay system. It has been shown previously¹ that L-glutamate reduced the inhibition but D-glutamate had no effect. Apparently the oxalacetate formed in a partially inhibited system can be removed by transamination with L-glutamate. The only mechanism which is compatible with these observations involves the reduction of the calcium ion concentration by tocopheryl phosphate, resulting in lack of activation of the DPN-ase system and subsequent formation of oxalacetate in the DPN-linked malate system. This type of mechanism of tocopheryl phosphate inhibition becomes important only in those systems in which the presence of a metal ion is essential to achieve maximum activity. Reduction of the metal ion concentration by formation of an insoluble salt with tocopheryl phosphate will result in an observed inhibition independent of any direct inactivation of the enzyme.

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SOME CHEMICAL AND ENZYMIC ALTERATIONS IN MUSCLES IN EXPERIMENTAL DYSTROPHY*

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A relation of α -tocopherol to cellular oxidation has long been suspected because of the high rate of oxygen consumption by muscle from animals on vitamin E-deficient diets. Confirmation of increased Q_{O_2} values was obtained from muscle strips of dystrophic rabbits, guinea pigs, and hamsters. This was most pronounced in rabbit muscle (Q_{O_2} , 1.9, vs. control, 1.3), but it was evident in guinea pigs, and also in hamsters that showed no external signs of dystrophy although they had been maintained on the deficient diet longer than is usually necessary to produce it (Q_{O_2} , 3.1, vs. control, 2.3). Age was not a factor, since the control and experimental hamsters from the same litter were used within a few days. The addition of the diffusible substrates, glucose and l-phenylalanine, to the Ringer's solution was without effect. Apparently, even in dystrophic muscle, adequate amounts of substrate are present.

Tocopherol phosphate has been shown to reduce the activity of the succinic dehydrogenase system in preparations from normal and dystrophic tissues alike.¹ Its action may be direct or indirect. By precipitating calcium below the level of concentration necessary to activate diphosphopyridine nucleotidase, it would preserve diphosphopyridine nucleotide (DPN), which inhibits succinic dehydrogenase,² or it might inhibit DPN-ase directly,³ thus protecting DPN. This has been shown to occur in the lactic acid dehydrogenase system in heart muscle.⁴ In either case, the supply of DPN would be decreased in vitamin deficiency. To determine whether the reduction of this component is related to the change in oxygen consumption, DPN was included in the substrate for respiring muscle strips. No influence on Q_{O_2} values was noted over a period of two hours. It is possible that DPN may not readily diffuse into muscle cells.

Since the rôle of tocopherol in the prevention of experimental dystrophy seems to be indirect, possible variations in other significant components of muscle tissue were sought.

If tocopherol is an antioxidant *in vivo* as it is *in vitro*, its absence might involve the disappearance of biotin, which has been shown to be destroyed in the presence of auto-oxidizing unsaturated fats. However, microbiological determinations, with *Lactobacillus arabinosus*, of both free and total biotin showed no variation, within the limits of error of the method, between normal and dystrophic muscles of hamsters, guinea pigs, and rabbits.† When the dietary intake of biotin by hamsters was lowered by the inclusion of egg white in the ration, muscle biotin was also decreased, irrespective of the vitamin E intake. Furthermore, dystrophy did not occur more rapidly when the biotin intake was low. If fatty acid peroxides are present in

* Supported in part by a grant-in-aid from the John and Mary R. Markle Foundation.

† These experiments will be reported in greater detail elsewhere.

dystrophic tissue, they do not destroy biotin *in vivo*, perhaps because of lack of contact or because biotin is combined with stabilizing substances.

Although the nature of the substrate undergoing increased combustion in dystrophic muscles is not known, the diminished creatine content of such muscles and the creatinuria, together with a considerable increase of creatine in the liver,⁵ tend to confirm the view that vitamin E may be linked with the metabolism of protein. If there is an abnormal oxidation of muscle protein in vitamin E deficiency, this might be reflected in the level of free amino acids in muscle tissue. Aminoaciduria has been demonstrated in patients with progressive muscular dystrophy.⁶ Since skeletal muscle contains a large proportion of its free amino acids as glutamine, variation in the amounts of this neutral storehouse of labile amino groups might indicate the nature of the altered processes leading to functional impairment. Glutamine was measured by the method of Hamilton.⁷

As shown in TABLE 1, the glutamine content of the skeletal muscle of dys-

TABLE 1
GLUTAMINE IN MUSCLE CARBOXYL-N

	<i>Non-glutamine</i> mg./100 g.	<i>Glutamine</i> mg./100 g.
Guinea pigs, +E (6)*	21.7	4.8
Guinea pigs, -E (7)	20.8	1.5
Rabbits, +E (6)	26.7	8.7
Rabbits, -E (5)	27.6	6.7

* Number of animals in the average.

trophic guinea pigs was strikingly decreased. In rabbits, the change from normal was less marked, perhaps not significant. This species difference may be due to the fact that on a vitamin E-deficient diet, rabbits become dystrophic within 2-4 weeks, whereas guinea pigs require 6-7 weeks. The total non-glutamine amino acid content of the muscles was influenced little, if at all, by vitamin E deficiency. A study of the distribution of other amino acids in this condition might be revealing.

The function of glutamine is still subject to speculation. Isotopic nitrogen fed to animals as ammonia or as amino acid is recovered largely as amide nitrogen in tissue proteins.⁸ It is the source of a large part of the urinary ammonia in acidotic dogs⁹ and serves as a neutral transport and storage form of labile amino groups for protein synthesis either directly or after transamination. The synthesis of glutamine is an endothermic reaction, as is the phosphorylation of creatine, which has been shown to be diminished in dystrophic muscle.¹⁰ Elliott¹¹ removed the adenosine triphosphatase activity from an enzyme system obtained from sheep brain. The resulting extract, plus glutamic acid, NH_3 , and adenosine triphosphate (ATP), produced glutamine and inorganic phosphate. The purified extract required Mg^{++} , and inactivation following prolonged dialysis could be reversed by cysteine. It was suggested that the $\gamma\text{-COOH}$ of glutamic acid is phosphorylated by ATP, the resulting compound then reacting with NH_3 to give inorganic phosphate.

The observed reduction in glutamine in the muscles of dystrophic animals may therefore be non-specific and indirect, resulting from a gradually decreasing supply of glutamic acid, of available amino groups, of glutaminase, of energy-rich phosphate in the form of ATP, or of diminished phosphorylation. Alternatively, the breakdown of glutamine to glutamate and NH_3 might be favored by the increased rate of respiration, which would remove α -ketoglutarate from the substrate.

Transamination has come to occupy a strategic position at the crossroads of many metabolic thoroughfares and particularly in the rebuilding of amino acids. Interferences with its normal rôle might well lead to the wastage of protein characteristic of dystrophy.

The reaction between aspartic and α -ketoglutaric acids to produce oxalacetic and glutamic acids was investigated by the manometric method of Green, Leloir, and Nocito,¹² as adapted by Ames and Elvehjem¹³ to the measurement of transaminase activity in tissue homogenates. However, the control flasks, instead of lacking α -ketoglutaric acid, contained the complete reaction mixture; the CO_2 released at zero time represented that coming from tissue and reagents prior to transamination.*

As shown in TABLE 2, the transaminase activity of homogenates of dys-

TABLE 2
TRANSAMINASE ACTIVITY OF SKELETAL MUSCLE HOMOGENATES

	<i>CO₂ per 100 mg. wet wt. ul</i>	<i>CO₂ per 10 mg. dry wt. ul</i>	<i>CO₂ per mg. N ul</i>
Guinea pigs			
+E (11)*	505	289	242
−E (10)	231	136	123
Rabbits			
+E (10)	92	84	63
−E (8)	70	51	40

* Number of animals in the average.

trophic guinea pig muscle was less than half of normal muscle. Dystrophic rabbit muscle showed a similar but less striking diminution. These results are uniform, whether expressed on the basis of wet weight of the tissue or of dry weight or total nitrogen content of the homogenate. Thus, the altered transaminase activity is not a reflection of the gross changes in tissue structure and composition accompanying dystrophy. Age was not a significant factor, for the control animals were, as nearly as possible, of the same age as the experimental animals at the time of use.

In both species, the addition of 0.05 mg. of pyridoxal phosphate produced no change in the evolution of CO_2 from either of two homogenate concentrations. This amount of pyridoxal phosphate was such as to allow coenzyme-enzyme complex formation in the twelve minutes of the equilibration period, even in the presence of the aspartic acid substrate, and could, therefore, be expected to restore the enzyme activity if coenzyme concentration had been the limiting factor.¹⁴

* These experiments will be reported in greater detail elsewhere.

An apparent decrease in transamination might be occasioned by an increased rate of removal of oxalacetate by other reactions.¹⁵ TABLE 3

TABLE 3
DECOMPOSITION OF OXALACETIC ACID BY MUSCLE HOMOGENATES

Oxalacetic acid added mg.	Amount and concentration of homogenate ml.	% Recovery of added oxalacetic acid	
		normal	dystrophic
		guinea pigs	
1.6	0.5 (1:20)	84 (6)*	86 (3)
1.6	1.0 (1:10)	80 (3)	88 (3)
		rabbits	
0.8	0.5 (1:20)	89 (2)	89 (4)
1.6	0.5 (1:5)	74 (4)	83 (3)
1.6	1.0 (1:5)	58 (4)	70 (3)

* Number of animals in the average.

shows that a 1:20 homogenate of dystrophic guinea pig muscle allowed recovery of approximately the same per cent of added oxalacetic acid after the reaction period as did normal muscle. With a 1:10 homogenate of dystrophic muscle, the recovery of added oxalacetate was the same as with the 1:20 homogenate, whereas, with the normal tissue it was less, if anything, a result exactly opposite to that which would indirectly account for the diminished transamination.

The data on rabbits are more striking and further justify the conclusion that the reduction in transamination is not an artifact produced by the increased removal of oxalacetic acid by some alternative pathway. In the case of the higher concentrations of rabbit muscle homogenate, the recovery of oxalacetic acid was noticeably greater with dystrophic muscle than with normal muscle, indicating that normal muscle possesses a better mechanism for disposing of excess oxalacetate than does dystrophic muscle.

Until more is known about the mode of action of vitamin E in controlling muscle metabolism, an explanation of the decreased transamination observed in dystrophic muscle can be only speculative. The decrease may be due to an altered oxidation-reduction state of groups necessary for activity of the enzyme, or to a shift in the equilibrium conditions.

The blocking of amidation, resulting in lowered glutamine levels, could lead to a higher concentration of glutamic acid, and, if this cannot undergo the normal amount of transamination, glutamic acid might be disposed of by oxidative deamination. Conversely, because transamination is a means of removing α -ketoglutaric acid, any diminution of this process might also account in part for increased oxygen uptake. These changes would affect protein synthesis: protein anabolism might be decreased through loss of building units; or, to obtain these units, other mechanisms, such as oxidative deamination and reductive amination, might be utilized to a greater extent.

Summary

(1) The increased Q_{O_2} of dystrophic muscle strips from animals deficient in vitamin E is not influenced by the addition of certain substrates or of diphosphopyridine nucleotide to the nutrient medium in which the strips are respiring.

(2) The biotin content of muscle tissue from dystrophic animals does not differ from that of the muscles of control animals.

(3) The glutamine level of muscle from vitamin E-deficient guinea pigs and rabbits is decreased from that of normal animals.

(4) The transaminase activity of skeletal muscle homogenates from dystrophic guinea pigs and rabbits is lower than that of muscles from control animals.

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Discussion of the paper

J. P. HUMMEL (*Department of Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa*): The decreased transamination, as well as the other diminished enzyme activities heretofore observed, may be only a reflection of the fibrotic substitution for active muscle mass. Such a process could well obscure any accelerated metabolic reactions. The activity of an over-active enzyme system may appear to be diminished unless the basis of comparison is the active mass of remaining muscle.

W. M. GOVIER (*The Upjohn Company, Kalamazoo, Michigan*): Quastel has shown by means of his ferricyanide system that DPN can diffuse into cells of liver whole cell preparations (slices). It would seem that the effects seen by Dr. Roderuck might be altered if nicotinamide were added to the system as a means of preserving DPN from breakdown by DPNase.

S. R. AMES (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): Recently, some observations were made relating vitamin E to amino acid metabolism.¹ Urinary excretion of amino nitrogen (also ascorbic acid) in rabbits deprived of vitamin E is doubled within a week. This appears to

be an early indication of vitamin E deficiency preceding the onset of creatinuria by several weeks. It may result from a localized deficiency of vitamin E in rapidly metabolizing tissues such as liver and kidney.

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C. G. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, New York*): In connection with the decreased transaminase activity of muscles in E-deficient rats reported by Dr. Roderuck, it may be significant that pyridoxine deficiency greatly intensifies the muscle lesions in young adult rats fed vitamin E-deficient diets. Just before the war, we made a study of the muscles in rats subjected to chronic deficiencies of five of the vitamins, both singly and in conjunction with vitamin E. These experiments were done in the laboratory of Professor E. V. McCollum at Johns Hopkins.

As is well known, the foci of hyaline necrosis that are found in the muscles of rats, placed on a vitamin E-deficient diet at weaning and continued on this diet for some months, are very widely scattered indeed. It is sometimes necessary to look through many low-powered fields before one such lesion is found. There was no critical evidence that this almost insignificant muscle pathology in the young adult E-deficient rat might not also occur in many dietary diseases. However, a thorough search of the muscles of rats subjected for months to chronic deficiencies which terminated in acute symptoms failed to reveal a single area of hyaline necrosis in diseases of the following deficiencies: thiamine, riboflavin, pyridoxine, pantothenic acid, vitamin A, and protein. Therefore, it is clear that, as insignificant as these E-deficient lesions may appear to be, they are none the less specific and characteristic of E deficiency with respect to other avitaminoses.

Consequently, it is of more than passing interest that superimposing either pyridoxine, vitamin A, or protein deficiency upon vitamin E deficiency greatly intensified the severity of the muscle lesions in rats subjected to these dual deficiencies. Muscle damage frequently approached in extent that seen in vitamin E-deficient rabbits and guinea pigs. That these results indicate some relation between vitamin E, on the one hand, and pyridoxine, vitamin A, and protein on the other, is evident from the fact that deficiencies of thiamine, riboflavin, and pantothenic acid failed to intensify the lesions of E-deficient rats.

The experiments reported by Dr. Roderuck in which E deficiency resulted in a reduction of the transaminase activity of rat muscle, suggest, as do our own experiments, that E is intimately concerned with protein or amino acid metabolism. The experiments of Davies and Moore have established the fact that vitamin E preserves the liver stores of vitamin A and thus delays the onset of A deficiency. Our experiments show the converse of this relationship, namely, that vitamin A deficiency intensifies the symptoms (muscle lesions) of E deficiency, and lead one to suspect that vitamin A is also concerned in amino acid metabolism. It was to be expected that protein deficiency would also intensify the effects of vitamin E deficiency.

Choline, while preventing the fatty livers that were otherwise found in the protein plus E-deficient rats, did not reduce the severity of the muscle damage. None of the rats used in these experiments showed signs of paralysis even after 30 weeks on the deficient diets.

Although Zenker's degeneration of the striated muscle is found in several infectious diseases, it would appear from these results that the necrosis of even occasional muscle fibers in diseases of nutritional origin is pathognomonic of E deficiency. Moreover, since an uncomplicated deficiency of a single vitamin seldom occurs outside the laboratory, it is probable that the muscle lesions of E deficiency will be greatly intensified as the result of the concomitant deficiency of other vitamins and protein. Blackfan and Wolbach¹ have reported muscle lesions in A-deficient infants. It seems likely that these babies were suffering from a multiple deficiency of vitamins A and E and perhaps other vitamins. These results probably represent one of the few accounts on record of E deficiency in the human.

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P. D. BOYER (*University of Minnesota, St. Paul, Minnesota*): In relation to Dr. Mackenzie's discussion, I would like to mention that some six years ago, in comparisons of the oxidation of pyruvate by dystrophic and normal rat-muscle minces, it was found that the amount of phosphocreatine formed in the presence of excess creatine was markedly less in the mince from dystrophic muscle. The oxygen uptake of the dystrophic muscle mince was equal to or greater than that of the normal muscle. However, the number of observations was small and the data have not been published. The results do give some support to the hypothesis that tocopherol might function in the generation of high-energy phosphate bonds formed during the oxidation of pyruvate. Experiments designed to test the validity of this hypothesis may yield worthwhile results.

ALPHA-TOCOPHEROL, ALPHA-TOCOPHERYL PHOSPHATE, AND PHOSPHORYLATION MECHANISMS

By Wm. M. Govier and Anna J. Gibbons

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Early in the serious study of vitamin E, some investigations^{1,2} pointed to the possibility that some form of α -tocopherol may be concerned in phosphorylation processes. This hypothesis, without doubt, was fostered by the facts that, in many animals, muscular dystrophy is the result of vitamin E deprivation, that muscle creatine is markedly lowered in muscular dystrophy, and that phosphocreatine is intimately connected with muscle contraction. However, the actual lowering of muscle phosphocreatine content has been placed in doubt, Lu, *et al.*³ having found no change in muscle phosphocreatine levels, whereas Morgulis and Spencer¹⁹ reported substantial decreases therein. Both laboratories reported decreased total acid-soluble phosphorus.

To make the rôle of vitamin E even more non-specific with regard to phosphorylation processes, Lu reported that glycogen phosphorylation was decreased by 46 per cent in muscular dystrophy, whereas Boyer⁴ found that dystrophic muscle could not utilize high-energy phosphate when certain Krebs cycle intermediates were oxidized. Torda and Wolff,⁵ considering acetylcholine synthesis to be driven by energy-rich phosphate, relate the increase in acetylcholine synthesis seen on addition of vitamin E to increased phosphorylation produced by α -tocopherol.

All of the work to date has been greatly hampered by the lack of knowledge as to the physiologically active form of vitamin E. Tocopheryl phosphate has been employed in much of the *in vitro* work because it is water-soluble and because Houchin and Mattill⁶ originally used it in their *in vitro* studies of the influence of α -tocopherol on oxidative systems. The evidence in favor of α -tocopheryl phosphate as the active form is meager. Karrer and Bussman⁷ believe it to be physiologically more active than the free alcohol. Morgulis and Jacobi⁸ have postulated that the phenol-phosphate linkage may be energy-rich, of the twelve kilogram calorie type, but, even if this were true, it would not necessarily mean that α -tocopheryl phosphate can be found normally in tissues. Houchin⁹ mentioned that tissues can dephosphorylate α -tocopheryl phosphate, and we have seen this phenomenon in our laboratory; but numerous examples exist of the dephosphorylation of an unnatural substrate by tissue phosphatases. Morgulis and Jacobi also postulated that α -tocopheryl phosphate may control phosphorylations by regulating ATPase, by reducing the excess of calcium available for activation of this enzyme in E deficiency. Hummel¹⁰ found, however, that the bulk of the excess calcium found in dystrophic muscle is in a non-ionizable form, and is not available for activation of ATPase.

Thus the study of tocopherol and tocopheryl phosphate would seem to be divided into at least two lines of attack: first, an effort to demonstrate a failure of phosphorylation in tocopherol-deficient animals; and second, an

attempt to show that some form of tocopherol *in vitro* can restore normal phosphorylation. The work of Hummel¹⁰ is in point on both cases. He was able to show that skeletal muscle homogenates from dystrophic animals were hampered in their ability to phosphorylate creatine if fructose 1,6-diphosphate or glycerophosphate were offered as substrates. He was, however, unable to show a return to normal on the addition of α -tocopheryl phosphate *in vitro*.

In our laboratory, we have made a preliminary study of guinea pig heart, employing the same system used by Hummel. The work is incomplete and has been beset with difficulties in handling guinea pigs on the diet used by Hummel, due to hair-eating and cannibalism, but a few experiments are presented in order to set out the problem. The diet of Basinski and Hummel¹¹ was modified by including only tocopherol-free lard as a source of fat. Control animals were given 7.5 mg. of α -tocopherol twice weekly. The *in vitro* system was unchanged, although the presence of magnesium ions would make it somewhat unsuitable according to Ames¹² because of the possibility of precipitation of α -tocopheryl phosphate as the magnesium salt. We found, however, that if the α -tocopheryl phosphate was added after all of the other components, no precipitation occurred. It should also be noted that, whereas Hummel found no wrist-stiffness in guinea pigs on this diet, both our E-deficient and control animals showed this phenomenon.

The experiments were set up in simple constant volume manometers, the vessel contents of which are shown in TABLE 1.

TABLE 1

Guinea pig heart homogenate, 10 per cent in water	1.0 ml.
Sodium phosphate buffer, 0.01 M, pH 7.4	0.1 ml.
KCl, when added	400 μ Moles
MgCl ₂	20 μ Moles
Nicotinamide	20 μ Moles
DPN	0.75 μ Mole
ATP, Na ₄	1.33 μ Moles
Sodium β -glycerophosphate	100 μ Moles
Cytochrome c, 4×10^{-4} M	0.1 ml.
Creatine	30 mg.
Sodium dl α -tocopheryl phosphate	0, 0.1, or 1.0 mg.
Water to make	3.0 ml.

The hearts of two to four animals were pooled for each experiment. The reaction mixtures were added to iced vessels, then equilibrated ten minutes at 37°, after which the manometers were closed and the reaction allowed to proceed for ten minutes. The reaction was stopped in control vessels at zero time and in the experimental vessels after ten minutes by the addition of 2.0 ml. of 17.5 per cent trichloroacetic acid. Phosphocreatine was determined in the filtrates by Potter's¹³ modification of the Fiske and SubbaRow procedure, using amidol as reducer,¹⁴ and is expressed in the table as μ Moles/vessel/hour.

Early in these experiments it was noted that the inadvertent omission of potassium led to a slight increase in synthesis of phosphocreatine when α -tocopheryl phosphate was added. When potassium was included, α -to-

copheryl phosphate produced a uniform lessening of phosphocreatine synthesis. TABLE 2 shows the results obtained in fifteen such experiments. It

TABLE 2
TOTAL PHOSPHOCREATINE FORMED, IN μ MOLE/VESSEL/HOUR

<i>E-Sufficient</i>			<i>E-Deficient</i>		
<i>Exp. No.</i>	<i>K</i>	<i>K + 1 mg. α-TPh/vessel</i>	<i>Exp. No.</i>	<i>K</i>	<i>K + 1 mg. α-TPh/vessel</i>
215	1.93	1.51	262	1.48	1.45
220	1.88	1.48	232	1.53	1.31
223	1.81	1.07		<i>K</i>	<i>K + 0.1 mg. α-TPh/vessel</i>
229	2.20	1.10			
			259	2.20	2.25
	<i>K</i>	<i>K + 0.1 mg. α-TPh/vessel</i>		<i>No K</i>	<i>No K + 0.1 mg. α-TPh/vessel</i>
235	2.68	2.42	244	1.31	1.83
265	1.40	1.61	247	1.12	1.35
			253	0.89	1.42
	<i>No K</i>	<i>No K + 0.1 mg. α-TPh/vessel</i>	256	1.23	1.60
				Av. 1.39	
238	1.84	5.47			
250	1.46	1.67			
	Av. 1.90				

will be noted that, judged by the average values for the vessels not containing tocopherol, deficient heart muscle may show a slightly impaired ability to phosphorylate creatine. There are not sufficient experiments for statistical analysis.

It may be seen in the table that in all cases the combination of K and 1 mg. α -tocopheryl phosphate (α -TPh) caused a small but distinct lessening of phosphocreatine accumulation. At the other extreme, the omission of K and addition of 0.1 mg. α -TPh produced a consistent small increase in phosphocreatine. In the middle groups, addition of K to the vessels already containing 0.1 mg. α -TPh greatly decreased or abolished the stimulation of phosphocreatine synthesis.

Although we have not entirely eliminated the possibility of interference in this system by ATP-ase, similar experiments in this laboratory in which ATP-ase activity has been measured in guinea pig heart under these conditions have shown a slight inhibition of ATP-ase activity by α -tocopheryl phosphate, the magnitude of inhibition being too small to be considered significant.

The rôle of potassium ions in this connection is an obscure one. The myo-

cardial lesions of vitamin E deficiency¹⁵ and those of potassium deficiency,¹⁶ the latter being ameliorated by thiamin deficiency,¹⁷ might seem to be somewhat related. The ambiguous place of thiamin in this picture is also apparent when one considers the relief of E-deficient dystrophy by massive doses of thiamin.¹⁸ Any attempt, however, to explain these phenomena would be pure speculation and must await further work.

Although in view of the wrist stiffness seen in these animals we cannot be sure that E deficiency plays a part in any of these results, we consider the importance of this work, if any, to lie in the promotion of phosphorylation by α -tocopheryl phosphate *in vitro*.

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Discussion of the paper

J. P. Hummel (*Department of Biochemistry, College of Medicine, State University of Iowa, Iowa City, Iowa*): The beta-glycerophosphate which Govier used as the substrate is poorly oxidized by muscle preparations as compared with alpha-glycerophosphate. This might account for the low rates of respiration and phosphorylation which he has reported.

K. E. MASON (*Department of Anatomy, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.*): The report of Holmes, claiming a beneficial effect of thiamine in late weaning paralysis of rats, could not be substantiated by studies carried out some years ago by Dr. Roger Terry in my laboratory; hence, I think that we can rule out possible interactions between thiamine and tocopherol. However, the effect of high thiamine on the EKG of our monkeys has not been tested.

INHIBITION OF HYALURONIDASE BY TOCOPHERYL ESTERS

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Introduction

The study of the mucopolysaccharide hyaluronic acid and of the enzyme, hyaluronidase, has resulted in a very large volume of work. This was pointed out some years ago by the excellent review of Duran-Reynals,¹ more recently in one by Meyer,² and most recently in a Conference at the New York Academy of Sciences last December 3 and 4.

Hyaluronic acid has been considered, from indirect evidence, to be the chief ground substance of mesenchymal tissue. It has been isolated from such diverse sources as connective tissue, bovine vitreous humor, groups A and C hemolytic streptococci, certain tumors in humans and fowl, and from rabbit and pig skin.^{1, 2} Our hyaluronic acid was prepared from human umbilical cords and was extensively purified by the method of Seastone.³ Certain preparations of hyaluronic acid have been considered to have a molecular weight of 200,000 to 500,000,⁴ with the estimate for the native form as one of several million.⁵

Hyaluronidase also occurs widely.^{1, 2} Among the sources are certain bacteria, leeches, the venoms of bees and of certain poisonous snakes and, in the animal body, in extracts of skin, spleen, ciliary body, and iris, as well as in aqueous humor from eyes of freshly killed cattle. While there are conflicting reports for its occurrence in some parts of the body,^{1, 2, 6, 7} there is no question concerning hyaluronidase in the testes. Our preparations were from bulls' testes and were semi-purified, corresponding to solution I of Hahn.⁸ Testicular hyaluronidase, in addition to depolymerizing hyaluronic acid, has been reported to attack chondroitin sulfate, a material which occurs with hyaluronic acid in many parts of the body. In contrast, streptococcal hyaluronidase has not been shown to attack chondroitin sulfate.²

The *in vivo* effect of hyaluronidase has been studied by injecting the enzyme along with a dye or India ink which then becomes spread over the affected area. This spreading effect is considered due to increased permeability caused by the depolymerization of substrate by the enzyme. In addition to skin, Duran-Reynals¹ has reported spreading in striated muscle, stomach, intestine, uterus, mammary gland, pancreas, mesentery, ovary, and the testicle itself. He also reported that testicular extracts markedly increased the permeability of the vascular system, although others have reported different results.²

The hyaluronidase system has been implicated in phenomena as varied as bacterial invasions and conception,¹ cancer,⁹ and rheumatic fever.^{10, 11} We hoped that a powerful inhibitor of this system might be a valuable chemotherapeutic agent at some point or, at least, that it might be a useful tool for eliminating some of the confusing claims arising in the hyaluronidase field. Could fate have been more unkind, then, than to have led us into the

Vitamin E field? However, as a result of our work, we think that one important function of Vitamin E or its derivatives may be to serve as regulators of hyaluronidase activity. When the concentration of regulator drops below a certain critical concentration in the body then the enzyme could attack its substrate.

Experimental

Methods. In our program, carried out after a rather broad literature survey, we have evaluated nearly one hundred compounds which we felt, for reasons sometimes obvious and sometimes obscure, might be likely to inhibit our enzyme system. The most effective inhibitors among the first seventy-five compounds tested are shown in TABLE 1.

TABLE 1
EFFECTIVE INHIBITORS OF HYALURONIDASE
Inhibitor, 1 mg. Hyaluronic Acid and 2 mg. Sodium Chloride per cc. M/60 Citrate-Phosphate Buffer at pH 7.0; Enzyme Added at Zero Time (Method I)

Compound	Concentration μg./cc.	Inhibition factor $\frac{T_I - T_C}{T_C}$
Heparin (Lederle's solution of sodium salt)	10	0.5
Aerosol 22 (N-Octadecyl-N-disodium succino disodium sulfosuccinamate)	25	0.5
Germanin (Bayer 205)	25	0.45
d,l-α-tocopheryl phosphate (Hoffman-La- Roche)	5	0.4
Tannic acid	2	0.5

Heparin was used as a reference compound because it has been used by several investigators since it was shown to be inhibitory by McClean some years ago.¹² It is a sulfonic ester of a polysaccharide. Aerosol 22 and Germanin also have sulfonic groups. The α-tocopheryl phosphate was chosen because it could possibly exert effects somewhat similar to Germanin and Heparin in the blood coagulation picture.¹³ Tannic acid on a weight basis was most active and, because the inhibitory activity of its solution increased on standing, each solution tested was freshly prepared. The inhibition factor is as defined, where T_C is the time for the control mixture to reach half viscosity and T_I is the time for the mixture with the inhibitor. Thus, for Heparin, T_I was in the vicinity of 18 minutes and T_C was around 12 minutes, so $\frac{18-12}{12} = 0.5$. We liked to have this factor around 0.5 but didn't always quite arrange it, as will be shown.

In obtaining these results, the enzyme was added at zero time to the inhibitor-substrate mixture. For a screening procedure, this is preferable to incubating the enzyme with the inhibitor. First, it should minimize any denaturation effects of larger concentrations of the compound on the enzyme. Second, it allows one to observe possible effects of compounds on the substrate. Upon addition of about 1μg. of the enzyme for each cc. of reaction

mixture, activity was followed by noting the reduction in viscosity of the reaction mixture. A typical uninhibited reaction by this method is indicated in Curve I (FIGURE 1). The endpoint, taken at relative half viscosity, is

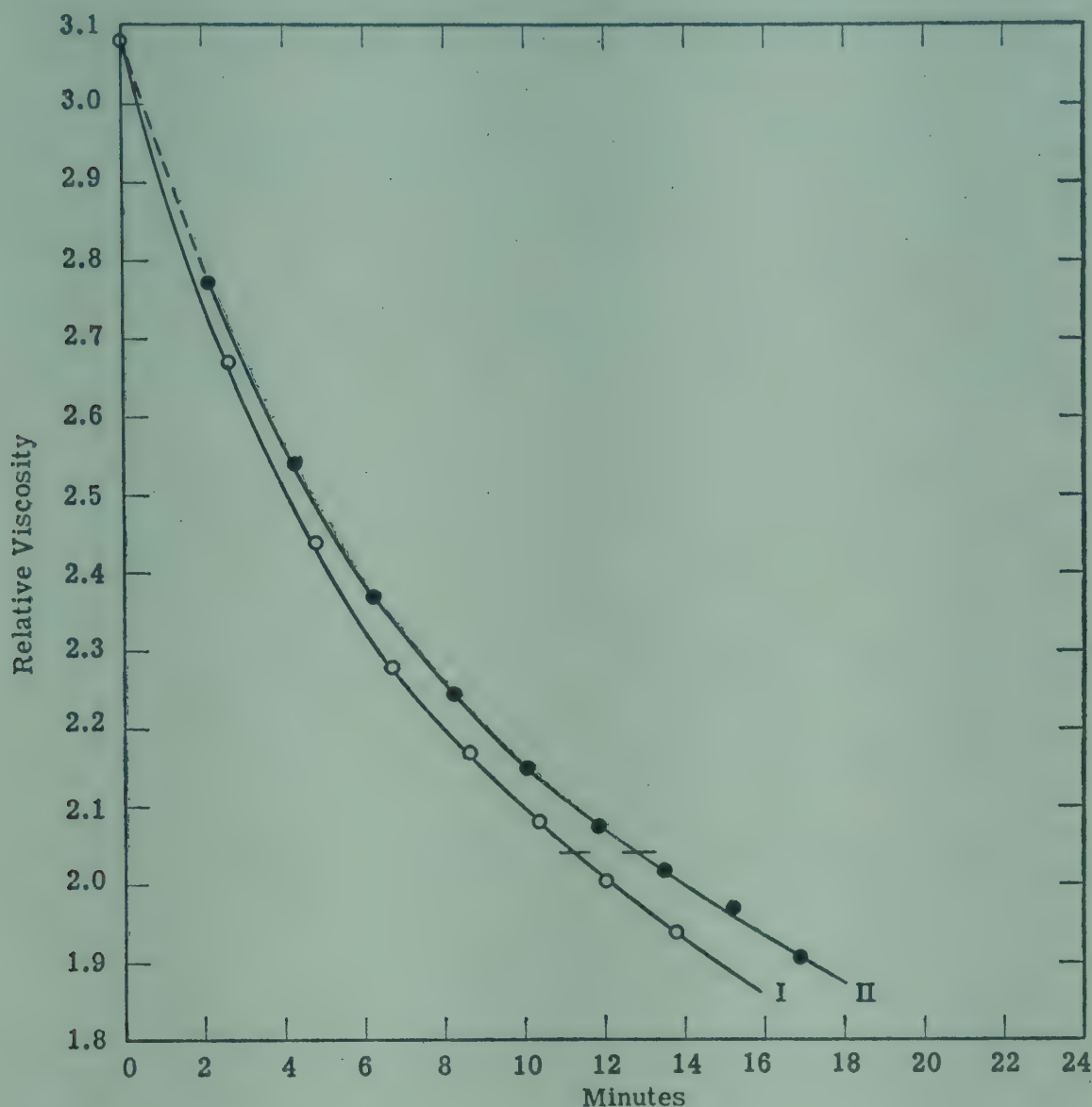


FIGURE 1.

Curve I—a total reaction mixture of 4 cc. prepared as indicated in TABLE 1, and 3 cc. of this used to determine the relative viscosity of the mixture. To 3 cc. in a test tube in the bath at 37.5°, enzyme solution (usually 0.06 to 0.1 cc. of 0.5 per cent gum arabic in M/60 buffer—not enough to alter viscosity appreciably) was added, and the whole mixed with a plunger and added to a second Ostwald viscosimeter of nearly equal flow time (41–43 seconds).

Curve II—enzyme in buffer added to 3 cc. of solution to give a final concentration of each reactant (except the enzyme) as for Curve I. After mixing, 3 cc. of mixture pipetted into viscosimeter. Point for zero time necessarily depends on consistency in compounding the reaction mixture similar to that for Curve I.

the time at which the viscosity becomes half of the distance from 1 to the starting viscosity—in this case just over 2. Our best working range was for endpoints from 10 to 20 minutes. In Method II, (Curve II of FIGURE 1) the enzyme diluted with buffer stood together for 2 minutes at 37.5° and was then added to the rest of the reaction mixture. A small inactivation of the enzyme is found. With this procedure, when various inhibitors are added to the enzyme buffer solution, their effects on the enzyme can be differentiated, while possible substrate effects are minimized.

The Effects of Anionic Surface Active Agents. In TABLE 1, Aerosol 22 was shown to be a good hyaluronidase inhibitor. It is an anionic surface active agent. By contrast, cationic and non-ionic surface active agents did not inhibit the hyaluronidase system. Aerosol SE, a stearic acid quaternary ammonium cationic type, and Tween 80, a polyoxyalkylene derivative of sorbitan mono-oleate and a non-ionic type, both caused the endpoint to

appear somewhat faster than for the control. Possibly, these compounds displace some enzyme molecules from the surface of the reaction mixture, thus protecting them from inactivating surface effects in the viscosimeter. These results contrast with those for the succinic dehydrogenase system, where anionic, non-ionic, and cationic type compounds were found inhibitory.¹⁴

The anionic surface active compounds might exert effects in the hyaluronic acid-hyaluronidase system primarily on the enzyme or the substrate or possibly both, depending on the circumstances. Some indication of this might be obtained by determining inhibitory concentrations using the two methods of FIGURE 1. The results with several similar compounds using Method I (adding the enzyme at zero time) are shown in TABLE 2. Other

TABLE 2
THE EFFECT ON HYALURONIC ACID-HYALURONIDASE OF AEROSOL 22 AND RELATED COMPOUNDS (METHOD I)

Compound	Formula	Surface tension of aqueous solution 0.1% (dynes per cm.)	Inhibitory concentration		Inhibition factor $\frac{T_1 - T_c}{T_c}$
			$\mu\text{g./cc.}$	$\times 10^{-6} M$	
Hexadecyl sulfate	$\text{C}_{16}\text{H}_{33}\text{OSO}_3\text{Na}$	37	5	1.5	0.31
Lauryl sulfate	$\text{C}_{12}\text{H}_{25}\text{OSO}_3\text{Na}$	29	12.5	4.3	0.25
Aerosol 22	$\begin{array}{c} \text{CH}_2\text{---COONa} \\ \\ \text{CH---COONa} \\ \\ \text{CH}_2\text{---CON} \\ \quad \quad \\ \text{NaO}_2\text{S---CH---COONa} \quad \text{C}_{18}\text{H}_{37} \end{array}$	44	10	1.5	0.37
Aerosol 18	$\begin{array}{c} \text{CH}_2\text{---CONHC}_{18}\text{H}_{37} \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	39	13.3	2.9	0.22
	$\begin{array}{c} \text{CH}_2\text{---CONHC}_{18}\text{H}_{37} \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$		16.7	3.6	0.51
Aerosol 02-22	$\begin{array}{c} \text{CH}_2\text{---CON}(\text{CH}_2\text{---CH}_2\text{---CH}_2\text{OC}_8\text{H}_{17})_2 \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	30	125	21.5	0.46
N-sec. Hexyldisodium sulfosuccinamate	$\begin{array}{c} \text{CH}_2\text{---CONH---C}_6\text{H}_{13} \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	60	1000	310	No inhibition
N-butyl disodium sulfo- succinamate	$\begin{array}{c} \text{H}_2\text{C---CONHC}_4\text{H}_9 \\ \\ \text{NaO}_2\text{S---CH---COONa} \end{array}$	76*	6670	2500	0.22
Victor Wetting Agent 35B	$\begin{array}{c} \text{C}_2\text{H}_5 \\ \\ \text{O} \quad \text{OCH}_2\text{---CH---CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_3 \\ \diagup \quad \diagdown \\ \text{P} \\ \diagdown \quad \diagup \\ \text{NaO} \quad \text{OCH}_2\text{---CH---CH}_2\text{---CH}_2\text{---CH}_2\text{---CH}_3 \\ \\ \text{C}_2\text{H}_5 \end{array}$	31	1000	290	0.64

* Surface tension of water 76 dynes/cm.

things being equal, the shorter the hydrocarbon chain the less effective the inhibitor—as note the difference between hexadecyl and lauryl sulfates and Aerosol 18 and related compounds. This dependence on chain length for this activity is similar to that for other properties of surface active compounds¹⁵ and, incidentally, also for the activity of compounds related to α -tocopherol.¹⁶ The surface tension data appears to be of only incidental interest.

In TABLE 3 the inhibitory concentrations of the most active compounds are compared with values obtained using Method II, where the inhibitor and the enzyme have the first chance at one another for two minutes before addition to the substrate mixture. The most striking difference is that

TABLE 3

THE VARIATION IN CONCENTRATION OF INHIBITOR WITH HYALURONIDASE METHOD

Compound	Method I		Method II	
	Enzyme added at T_0		Enzyme and inhibitor added at T_0	
	Concentration inhibitor $\mu\text{g./cc.}$	$\frac{T_I - T_C}{T_C}$	Concentration inhibitor $\mu\text{g./cc.}$	$\frac{T_I - T_C}{T_C}$
Hexadecyl sulfate	5	0.31	1.25	0.6
Lauryl sulfate	12.5	0.25	0.5	0.44
Aerosol 22	10	0.37	0.25	0.62
Aerosol 18	16.7	0.51	0.33	0.40
Aerosol 02-22	125	0.46	2.5	0.37

less, and usually much less, inhibitor is required in Method II. Also, the ratio of amounts used in Method I and II for the different compounds varies somewhat.

However, certain compounds were found for which the same concentration was about equally inhibitory in both Methods I and II. Among our best inhibitors, Germanin and Heparin behave this way (TABLE 4). The same

TABLE 4

INHIBITORS AT THE SAME CONCENTRATION IN BOTH HYALURONIDASE METHODS

Compound	Concentration $\mu\text{g./cc.}$	Method I Enzyme added at T_0 $\frac{T_I - T_C}{T_C}$	Method II Enzyme and in- hibitor added at T_0 $\frac{T_I - T_C}{T_C}$
Heparin (Lederle's solution of sodium salt)	15	0.5	0.55
Germanin (Bayer 205)	20 or $1.4 \times 10^{-5} \text{ M}$	0.67	0.84

Each cc. of M/60 citrate-phosphate buffer at pH 7.0 contained finally in addition to inhibitor as indicated, 1 mg. hyaluronic acid and 3 mg. sodium chloride.

concentration does appear somewhat more effective in Method II, but any preferential effect on the enzyme in this method is very minor compared to the large differences shown with the compounds in TABLE 3. Heparin and Germanin may inhibit the hyaluronic acid-hyaluronidase system primarily by affecting the substrate and likely through some sort of combination with it. Despite larger implications, at least for this paper, this is one attractive explanation for the large differences in the concentrations of certain inhibitors using Methods I and II, provided that whatever happens between hyaluronic acid and Heparin or Germanin can also happen with these anionic inhibitors. In Method I, hyaluronic acid and inhibitor are together for several minutes before the enzyme is added. If, during this period, most of the inhibitor becomes associated with the substrate, then the amount free in solution for the enzyme is reduced, and possibly below even the small amount shown to be necessary to affect the enzyme in Method II. A further

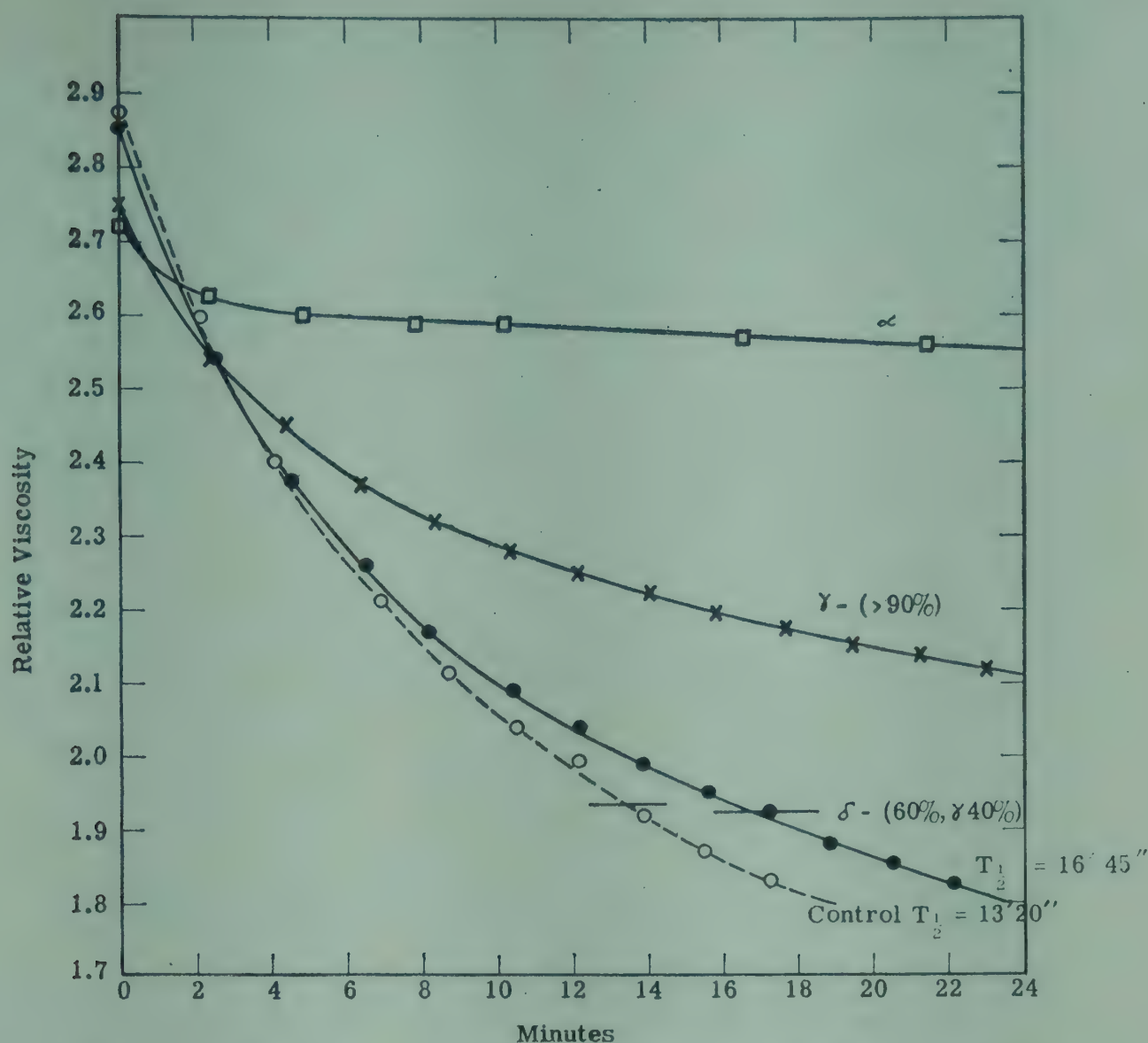


FIGURE 2. Showing the effect of 5.5 $\mu\text{g.}$ tocopheryl phosphates on the reduction in viscosity of hyaluronic acid by hyaluronidase.

using the d- γ -tocopheryl phosphate, and with the d- α -tocopheryl phosphate the reaction was slowed almost to a complete stop.

The differences in inhibitory concentrations of the different tocopheryl esters is much greater using Method II, where their effect is primarily on the enzyme (TABLE 6). The natural d- α -tocopheryl phosphate at 0.07 $\mu\text{g.}$ or 10^{-7} M is again somewhat more active than the synthetic dl-compound and both are more active than the succinate. The γ -tocopheryl phosphate is about $\frac{1}{3}$ as active as the α - even though more than twice as active as the δ -mixture. Indeed, in the 0.36 $\mu\text{g.}$ of δ -mixture there is 0.144 $\mu\text{g.}$ of γ -tocopheryl phosphate which might account for the most of the effect of the whole δ -mixture (compare with 0.18 $\mu\text{g.}$ of γ -compound in TABLE 6). The considerably larger difference in the relative inhibitory concentrations of the various tocopheryl esters in the hyaluronidase system, using Method II compared to the results with Method I, means that α -tocopheryl phosphate exerts a relatively greater specificity over these other esters when the effect is primarily on the enzyme protein rather than on the polysaccharide substrate. This suggests an *in vivo* relationship between Vitamin E and hyaluronidase, but this idea would be more attractive if α -tocopherol also could be shown to inhibit this enzyme.

In FIGURE 3 it appears that with Method II, which requires much smaller concentrations of inhibitor than Method I, an inhibitory effect can be dem-

TABLE 6
THE VARIATION IN INHIBITORY CONCENTRATION OF TOCOPHERYL ESTERS WITH HYALURONIDASE METHOD

Formula	Compound	Method I Enzyme Added at T ₀		Method II Enzyme and inhibitor added at T ₀		
		Conc. inhibitor μg./cc.	$\frac{T_i - T_c}{T_c}$	Conc. inhibitor		$\frac{T_i - T_c}{T_c}$
				μg./cc.	$\times 10^{-7} M$	
	dl-α-phosphate*	3.25	0.25	0.07	1.3	0.82
	d-α-phosphate*	3.25	0.38	0.07	1.3	0.95
	d-α-succinate	5.0	0.44	0.125	2.4	0.27
	d-γ-phosphate* (>90%)	4.0	0.44	0.18	3.4	0.48
	d-δ-phosphate* (δ 60%, γ 40%)	5.5	0.27	0.36	6.9	0.29

* Weighed as the disodium salt.

onstrated with d-α-tocopherol. The effect is not large nor is it proportional to the d-α-tocopherol added. Because the free tocopherol was so insoluble in the aqueous system employed, it was necessary to dilute it into the reaction mixture using a dioxane solution (which, when used alone, slows the enzymatic reaction somewhat. See FIGURE 3). The proportion of the tocopherol which was in effective solution was not determined, but it was probably only a relatively small amount. A Tyndall effect could be observed with these reaction mixtures as well as with those containing 2.5 μg. d-α-tocopherol per cc. Data not presented here indicated that the concentration of 2.5 μg./cc. appeared about as inhibitory as the concentration of 6.25 μg./cc., indicating a saturation phenomenon relative to d-α-tocopherol. Thus, while these results do not allow a comparison of the relative effectiveness of the phosphate ester to that of α-tocopherol, it is likely that the latter is exerting its effect at a quite low concentration.

Factors Affecting the Inhibition. It was stated earlier that Tween 80, a non-ionic surface active agent, did not inhibit the hyaluronidase-hyaluronic acid reaction but that the endpoint was somewhat faster than for the control reaction. While contemplating the possibility of using this material as a dispersing agent in our experiments with d-α-tocopherol, we wondered whether it might not interfere with any inhibitory effects of the vitamin. Accordingly, Tween 80 was tested for any effect on the inhibition caused by dl-α-tocopheryl phosphate. When only 2½ times the amount of Tween (relative to dl-α-tocopheryl phosphate) was added with the phosphate to the substrate mixture (Method I), no inhibition was noted upon adding the enzyme (TABLE 7). By contrast, when Method II was used, in which the

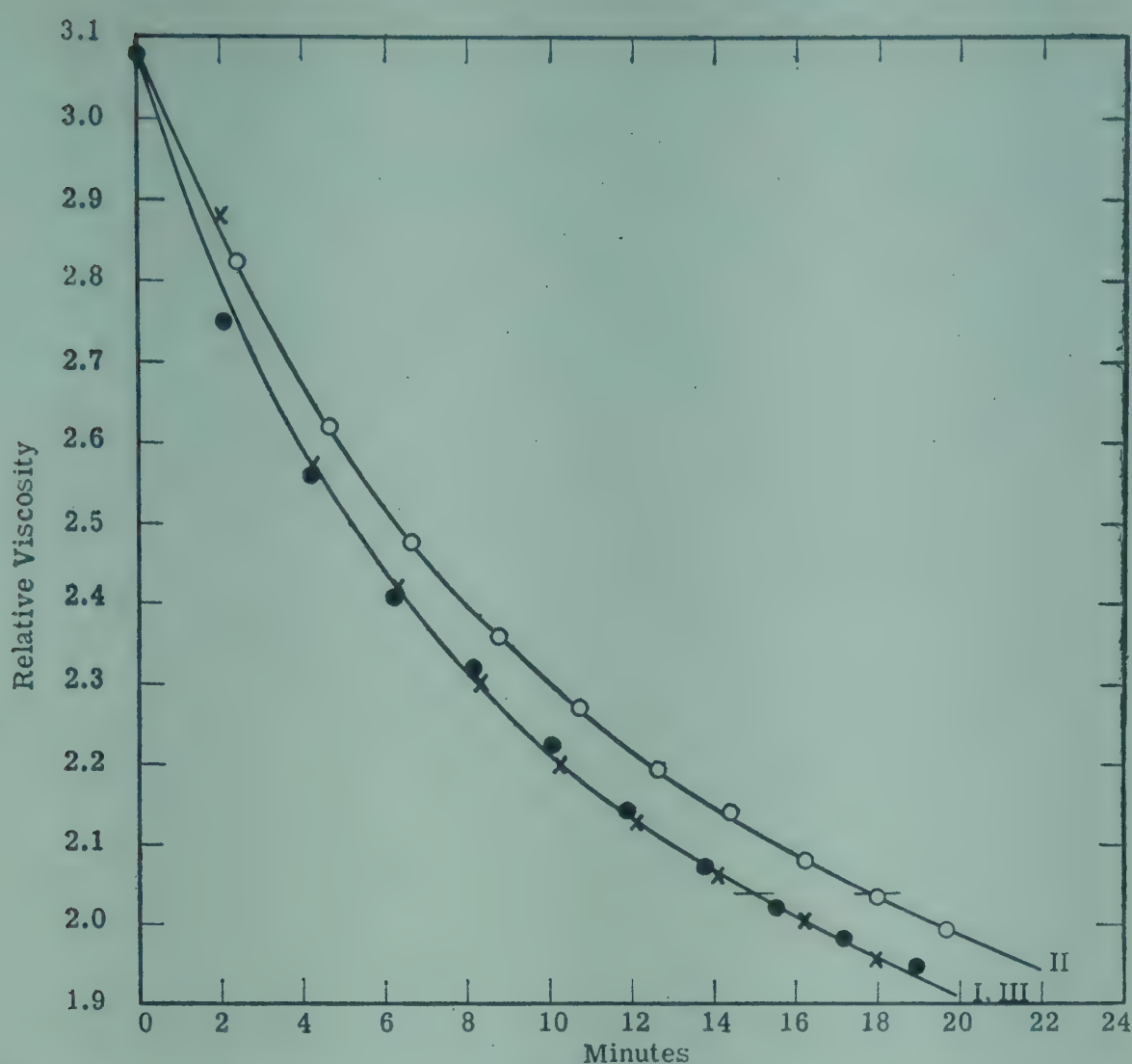


FIGURE 3. Showing the effect of α -Tocopherol (in Dioxane) on the hyaluronic acid—hyaluronidase system (Method II).

- I. \times —0.00625 cc. Dioxane/cc. $T_{\frac{1}{2}} = 14'55''$,
 II \circ —As I but with 6.25 μg . α -Tocopherol added/cc. $T_{\frac{1}{2}} = 17'45''$.
 III \bullet —As I but immediately after II Control. $T_{\frac{1}{2}} = 11'45''$.

enzyme was added to the Tween 80, dl- α -tocopheryl phosphate, and buffer mixture and allowed to stand for two minutes, it was found that with a Tween/dl- α -tocopheryl phosphate ratio of 2.5, the reaction was partially inhibited but, when this ratio was increased to 10, no inhibition was found.* That the Tween 80 in these experiments may prevent some sort of combina-

TABLE 7
FACTORS INFLUENCING HYALURONIC ACID-HYALURONIDASE INHIBITION

Compound	Conc. μg .	Tween 80 Conc. μg .	Procedure	$\frac{T_I - T_C}{T_C}$	Comment
Germanin	20	50	Add enzyme at T_0 (Method I)	0.52	Not an appreciable effect
	20	200	" "	0.13	Effect nearly reversed
dl- α -tocopheryl phosphate (synthetic)	3.25	8.14	" "	—	No inhibition; $T_C = 12' 48''$; $T_E = 12' 36''$
	0.07	0.18	Incubate enzyme with Tween and the α -phosphate 2 min. Add at T_0 . (Method II)	0.3	About $\frac{1}{3}$ of inhibition without Tween
	0.07	0.70	" "	—	No inhibition; $T_C - T_E = 45''$
	0.10	417	Incubate enzyme with α -phosphate for 2 min. Add at T_0 to Tween substrate mixture in buffer. (Method II)	0.32	More than half the inhibition removed.
	333	Bovine serum albumin μg . 1000	Add enzyme at T_0 to albumin, hyaluronic acid, tocopheryl phosphate mixture (Method I)	0.29	Hyaluronidase = about $1\mu\text{g}/\text{cc}$.

T_E = Time for experiment; T_C = Time for control.

* Since Tween 80 is sometimes used in vitamin E preparations, it would be valuable to know whether it can exert similar effects *in vivo*.

tion between the enzyme and the α -tocopheryl phosphate is suggested by the large increase in the amount of Tween necessary to relieve the inhibition observed after the enzyme and the α -compound have stood together for two minutes. With the Tween/ α -tocopheryl phosphate ratio increased to nearly 4200, only about half the inhibition was removed.

Germanin, which was about equally effective as an inhibitor in either Method I or II (see TABLE 4), was studied with Tween 80 using Method I (TABLE 7). In contrast to the experiment with dl- α -tocopheryl phosphate (Method I), with a ratio (Tween/Germanin) of 2.5, no appreciable effect on the inhibition was found and, even when this ratio was increased to 10, inhibition was still apparent. Tween 80 thus appears less effective in interfering with inhibition due to Germanin, where the effect is presumed to be primarily on the substrate, than with α -tocopheryl phosphate, whether the effect is on the substrate (Method I) or on the enzyme (Method II).

In TABLE 7, using Method I, the influence of crystalline serum albumin on the inhibition of hyaluronidase by dl- α -tocopheryl phosphate is shown. When this protein was added in about 1,000 times the amount of enzyme, the α -tocopheryl phosphate necessary for inhibition was increased only 100-fold. Some increase in the amount of this compound required under these conditions might be expected since, as indicated by Ames and Risley,¹⁷ serum albumin combines with it. However, the question of specificity of the effect of α -tocopherol or its phosphate ester in the hyaluronidase system will be better answered by a determination of the relative inhibitory concentrations of these compounds in other enzyme systems.

Discussion

To associate the hyaluronidase system *in vivo* with vitamin E, a direct correlation of the effectiveness of the various tocopherols on the enzyme system with their known biological activities might be ideal evidence. Yet, to go from the viscosimeter to the animal is quite an extrapolation, and in this paper it is necessary to use the phosphate esters rather than the free tocopherols, although some inhibition had been demonstrated for α -tocopherol. Nevertheless, the agreement in the order of the values for biological activity of tocopherols and hyaluronidase inhibition by tocopheryl phosphates seems surprisingly good.

Considering the varying reports from the different laboratories, Mason¹⁶ indicates that the anti-sterility potency of α -tocopherol may be 2 to 4 times that of γ -tocopherol, with perhaps the best value about 8 times. In a similar test, δ -tocopherol was reported to have an activity less than $\frac{1}{100}$ that of α -tocopherol.¹⁸ As reported here, relative to their effect on hyaluronidase, the d- α -tocopheryl phosphate was about 3 times as active as the d- γ -compound and more than 5 times as active as the δ - γ -mixture. In fact, the activity of the latter could be due largely to the d- γ -tocopheryl phosphate present in the δ - γ -tocopheryl phosphate mixture, which leaves open the question of the activity of the δ -compound.

Several papers in this monograph attribute effects of α -tocopheryl phosphate in enzyme systems to its detergent properties not related to the bio-

logical action of vitamin E. While, as pointed out by Glassman,¹⁵ "the utility of any compound as a wetting agent, detergent, or emulsifying agent is an expression of an aggregate of properties including specific chemical configuration and is inadequately expressed by any one simple measurement such as surface tension lowering," it is interesting, nonetheless, to note again that for 0.1 per cent solutions only the δ - γ -tocopheryl phosphate mixture lowered the surface tension appreciably at pH 7, although the γ - and α -compounds exerted some effect. Yet, all of these satisfy the requirement for a surface active compound: a balance between polar and non-polar groups in the molecule. The same type of balance also exists in the free α -, γ -, and δ -tocopherols. While the phosphate ester is appreciably more hydrophilic (and likely more reactive, hence less specific) than the hydroxyl group, in either the phosphate ester series or with the various free tocopherols, the relative availability of the hydrophilic group to the solution should be similarly influenced by the configuration of methyl groups and would be expected to be the poorest with the α -tocopherol or its phosphate ester and the best with the δ -tocopherol or its phosphate ester. It would, therefore, seem possible that with some enzymes the relative activity of various phosphate esters might give some indication of the relative activity of the corresponding tocopherols. It is worth suggesting, in view of the influence of Tween 80 on the inhibition of hyaluronidase by α -tocopheryl phosphate, that perhaps the δ -tocopheryl phosphate inhibits less because it is more surface active than the α -compound.*

Because the inhibitory concentration of α -tocopheryl phosphate is low (10^{-7} M), because the order of activity of the various phosphate is in good agreement with the generally accepted biological activities of the various tocopherols, and because α -tocopherol also has been shown to have an inhibitory effect on the enzyme, there would appear to be good basis for suggesting that one important function of vitamin E may be to regulate hyaluronidase activity *in vivo*.

This suggestion also could offer one explanation for many of the effects observed in vitamin E-deficient animals, when one considers the spreading reaction caused by hyaluronidase in various tissues as described in the introduction. However, because there is no question as to the occurrence of hyaluronidase in the testes, some knowledge of the sequence of events there did much to encourage our thinking that, when E levels drop below a certain concentration, hyaluronidase may attack neighboring substrate. As regards E-deficient male rats, Mason¹⁶ has pointed out that there is no injury to the seminiferous epithelium prior to maturity. Maturity is characterized by the production of spermatozoa. These have been shown by several laboratories to contain considerable quantities of hyaluronidase.^{20, 21, 22} In other words, these E-deficient rats were doing all right until hyaluronidase from sperm appeared. (Extracts from immature testes show little spreading activity.¹) Evans and Burr²³ also have indicated that, at the first stage in the degeneration process, sperm lose their fertilizing power coincidentally

* Because of the emphasis which has been placed on vitamin E as an antioxidant,¹⁹ it is perhaps of interest that the relative activities of α -, β -, γ - and δ -tocopherols as anti-oxidants for vitamin A acetate in olive oil at 39° is 1:1.3:1.8:2.7.¹⁸ This is the reverse of the order for the biological potencies of the compounds.

with the first appearances of histological changes in the germinal epithelium. This could also indicate a disturbance in the hyaluronidase of the sperm, since it is more generally considered that only sperms with this enzyme can fertilize.² Thus, it would appear that, somehow, vitamin E may influence hyaluronidase behavior in the testes.*

Summary

Various tocopheryl phosphates have been shown to be inhibitors of hyaluronidase in the same relative order of effectiveness as generally accepted for the biological activity of the corresponding free tocopherols: $\alpha > \gamma > \delta$. Some inhibition of this enzyme has also been demonstrated with d- α -tocopherol. These facts support the idea that one important function of vitamin E may be to regulate hyaluronidase activity in the body.†

Acknowledgment. The authors are particularly grateful to Dr. Stanley Ames of Distillation Products, Inc. for helpful suggestions and for samples of d- α -tocopheryl phosphate, d- γ -tocopheryl phosphate and the d-(δ - γ)-tocopheryl phosphate mixture; to Mr. Emil Vitalis of the Textile Laboratories, Stamford Research Laboratories, American Cyanamid Company for valuable advice and the surface tension data here reported; and to the Hoffman-LaRoche Company for a sample of dl- α -tocopheryl phosphate. They are also indebted to Dr. Karl Mason and Dr. C. G. Mackenzie, as well as to several colleagues at the Stamford Research Laboratories, American Cyanamid Company.

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* Hyaluronidase appears less obviously implicated in the anti-sterility effects of vitamin E in the female rat, although certain aspects of the deficiency state suggest that this enzyme could be involved.²³ In this regard, the early work of Boyland and McClean, done using only the spreading reaction in rabbits as an indicator of activity,⁹ is of some interest. They considered that extracts of placental and embryonic tissue of normal rats contained "more of the diffusing factor than other normal tissues except the testes." It would seem worthwhile to check their observations, using these tissue extracts and purified hyaluronic acid in a simple *in vitro* medium such as employed in this work.

† This idea has recently been expressed by others—notably Burgess and Pritchard²⁴—but without supporting evidence.

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Discussion of the Paper

DR. G. C. DOWD (*Boston Evening Clinic and Hospital, Boston, Mass.*): The hyaluronidase activity of a semen sample of a patient receiving vitamin E therapy was negative. This was not found to be so prior to and after cessation of E therapeusis.

ON CERTAIN EFFECTS OF ALPHA-TOCOPHERYL PHOSPHATE ON OXIDATIVE MECHANISMS OF STRIATED MUSCLE*

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There is ample evidence, derived largely from observations of vitamin E-deficiency states, that α -tocopherol participates in the metabolism of skeletal muscle. In order to define this relationship further, we have studied, in normal rats, the effects of administration of a derivative of α -tocopherol on oxidative mechanisms in skeletal muscle and on the response of a nerve-muscle preparation of the intact animal.

Because it is relatively water-soluble, and, therefore, convenient to administer parenterally, d,l- α -tocopheryl phosphate disodium‡ (α -TPh) was the agent employed. It must be emphasized at the outset that α -TPh is not the same as free tocopherol and that phenomena attributable to it cannot, in strictest accuracy, be claimed for vitamin E. This reservation is

TABLE 1

ENDOGENOUS OXYGEN CONSUMPTION OF BRAIN AND LIVER SLICES. MEANS AND STANDARD DEVIATIONS

Tissue	Q _{O₂}	
	Normal	α -TPh
Brain	39.8 \pm 6.2 (7 rats)	39.3 \pm 7.8 (7 rats)
Liver	27.9 \pm 14.8 (5 rats)	27.7 \pm 8.3 (5 rats)

For experimental conditions see FIGURE 1. Q_{O₂} = μ l. O₂/100 mg. wet wgt./hour.

not entirely applicable to instances in which α -TPh has been administered to the intact rat. In such cases, there is reason to believe that significant dephosphorylation occurs¹ and that the active agent is indeed free α -tocopherol.

When α -TPh was administered to normal rats, subcutaneously or intraperitoneally, a single large dose produced, within a few to 30 minutes, a state of apparent drowsiness, ataxia, flaccidity, and, occasionally, death after several hours. When the rat was sacrificed 20 to 30 minutes after injection of α -TPh, the endogenous respiration of diaphragm (*i.e.*, the oxygen consumption of the tissues suspended in a buffered electrolyte solution) was reduced by about 25 per cent (FIGURE 1). The respiratory quotient of these diaphragms, whether or not the animal had received α -TPh, was very nearly 1.00. Contrary to the depression found in skeletal muscle, endogenous respiration of liver and brain slices was not affected by administration of α -TPh (TABLE 1).

* Work performed under a contract between the Office of Naval Research, Navy Department and the Johns Hopkins University and assisted by a grant-in-aid from the Committee on Therapeutic Research of the Council on Pharmacy and Chemistry, American Medical Association.

† With the technical assistance of M. Glass, D. Field, E. Leakins, and L. Knight.

‡ We are indebted to Dr. Leo Pirk of Hoffmann-La Roche, Inc., Nutley, New Jersey for generous supplies of α -TPh.

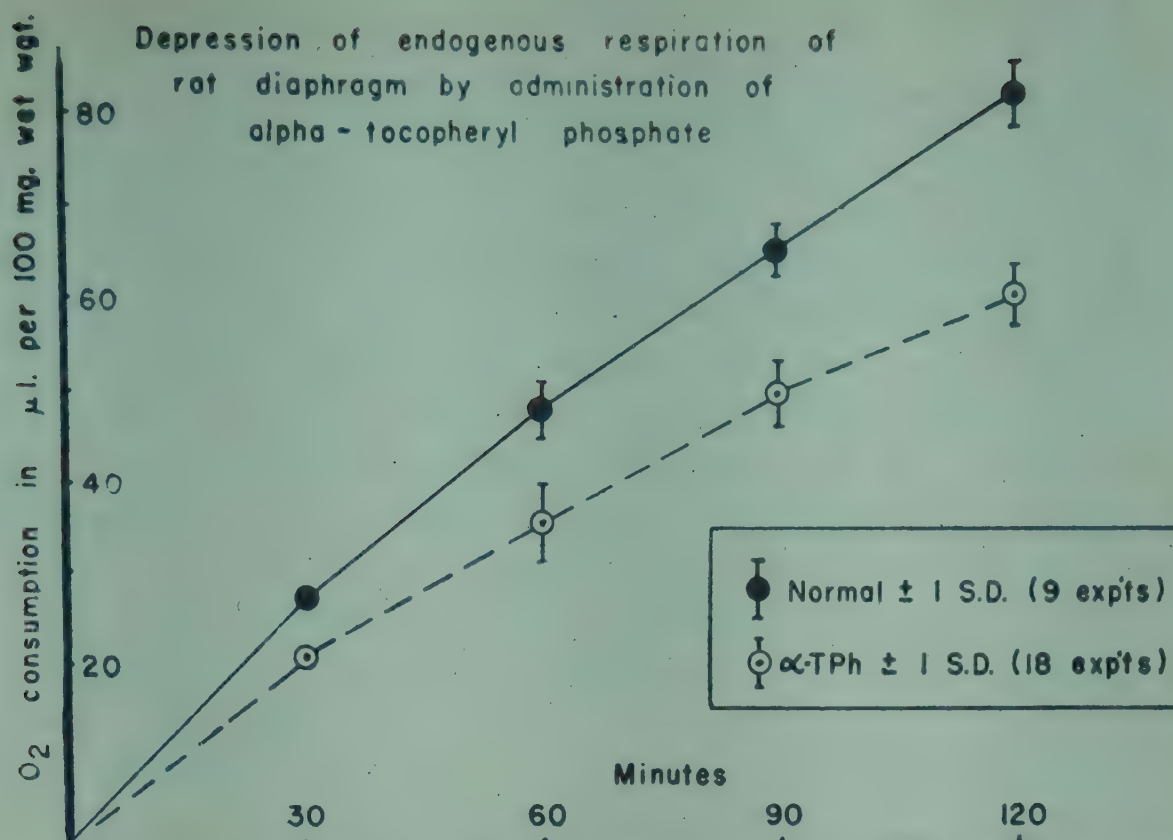


FIGURE 1. Sections of diaphragm, approximately 100 mg. each, were rapidly weighed and placed in conventional Warburg reaction vessels. Contents of flasks (final concentrations): 0.051 *M* NaCl, 0.003 *M* KCl, 0.001 *M* CaCl₂, 0.065 *M* phosphate buffer, pH 7.3. Final volume, 3.1 ml., including 0.1 ml. of 2 *N* NaOH in center well. Equilibration, 10 minutes. Temperature, 37° C. Atmosphere, air.

Since skeletal muscle provides nearly half the body weight, it might be anticipated that decreased oxygen consumption of the whole rat would accompany administration of α -TPh. The results of such an experiment are illustrated in FIGURE 2. Seven male rats from a single litter were divided into two groups, A (four rats) and B. For four weeks prior to exhibition of α -TPh, the basal oxygen consumption of the two groups was essentially the same. During the fifth week, the rats in group A received α -TPh in their drinking water (about 1 g./k. body wt./24 hours). Their basal oxygen consumption decreased by about 12 per cent, while that of untreated group B was unchanged. During the sixth week, the rats in group B received α -TPh and their basal oxygen consumption decreased by about 12 per cent.

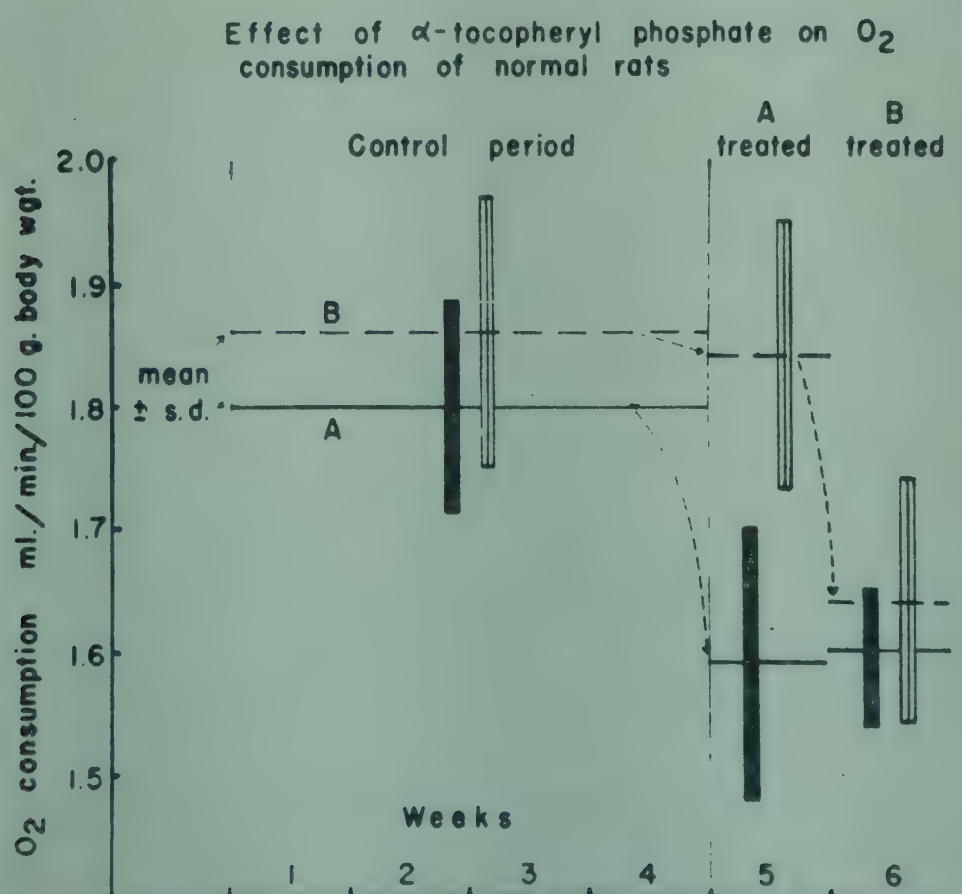


FIGURE 2. 7 male litter mates; 4 in group A, 3 in group B. Basal oxygen consumption of each rat measured by coiled volumeter technique⁶ three times a week.

Was reduction in oxygen consumption mediated by way of the thyroid hormone? This has seemed unlikely, since neither gross nor microscopic changes appeared in the thyroid gland* after 10 days of α -TPh administration and since α -TPh did not lessen significantly the increased oxygen consumption induced by thyroxin.

It is probable, then, that the depression in oxygen consumption of the intact rat which accompanies administration of α -TPh is largely, but not necessarily exclusively, a reflection of reduced oxygen consumption in striated muscle, and that this, in turn, is a consequence of derangement of a normally operative intramuscular respiratory system.

For a long time, efforts to characterize this derangement met with complete failure. Although a number of selected energy systems was assayed, no defect was found in these specific functions after administration of α -TPh. Thus, injection of α -TPh did not modify the following activities of skeletal muscle: succinoxidase, cytochrome oxidase, adenosinetriphosphatase, acid phosphatase, glycolysis, acetoacetate consumption, or proteinase (hemoglobin substrate).

Why does injected α -TPh, in doses sufficiently large to reduce the over-all consumption of oxygen, fail to inhibit systems *in vivo* which are inhibited by addition of α -TPh *in vitro*? This difference in behavior is explainable if one assumes that the widespread, and apparently non-specific, inhibitory activity of α -TPh added *in vitro* is largely the result of one property of that molecule, while the specific (because not widespread) inhibitory activity of injected α -TPh is the result of another property. What are these two properties?

The widespread inhibitory effects of α -TPh *in vitro* can be explained if one assumes that α -TPh is adsorbed on the surfaces of proteins; and, indeed, α -TPh is a detergent. It may be considered a member of the heterogeneous group of surface-active agents which Hockenhull² has reported inhibit rabbit muscle succinic dehydrogenase.

If α -TPh is dephosphorylated in the body, phenomena owing to its detergent property would not appear. Localization of the effects of injected α -TPh largely to a particular phase of muscle metabolism is consistent with this thesis, and it is suggested, therefore, that the observations to be reported are indeed the result of acute hypervitaminosis-E.

It will be recalled that α -TPh, administered to the rat, produced a fall in basal oxygen consumption in the intact animal and a reduction in the rate of endogenous respiration of skeletal muscle, but not of liver or of brain.

Since the respiratory quotient of striated muscle, during endogenous respiration, is very nearly 1.00, it is assumed that the bulk of oxygen consumption, under these conditions, involves the metabolism of carbohydrate. Significant depression of endogenous respiration, then, might be in consequence of impaired glycogenolysis or glycolysis. With glucose as substrate, however, there was no difference in oxygen consumption between diaphragms of normal rats and of α -TPh-injected rats.

* Dr. Richard Follis, Department of Pathology, The Johns Hopkins University and Hospital, examined the thyroids of these rats.

There remained the possibility of defective breakdown of glycogen to glucose. Glycogenolysis can be accelerated by administration of epinephrine. Immediately after exhibition of epinephrine to the normal rat, both liver and muscle glycogen are reduced sharply. After an hour or so, there occurs appreciable resynthesis of liver glycogen from muscle lactic acid.³ If injected α -TPh does not prevent liver glycogenolysis but does impede muscle glycogenolysis, administration of epinephrine to rats previously treated with α -TPh should result in relatively slight loss of muscle glycogen but relatively great loss of liver glycogen, since the supply of lactic acid available from muscle for resynthesis of liver glycogen would be inadequate.

This hypothesis was tested. Only preliminary data have been accumulated and it cannot be said that the hypothesis has been proven, although the results support it.

The first experiments were performed on two groups of six rats each (TABLE 2). α -TPh was injected into one group. Thirty minutes later both

TABLE 2

EFFECT OF α -TPh ON GLYCOGENOLYSIS INDUCED BY EPINEPHRINE. MEANS AND STANDARD DEVIATIONS

<i>Tissue</i>	<i>Mg. glycogen per G. tissue</i>	
	<i>Without α-TPh</i>	<i>With α-TPh</i>
Liver	23.3 \pm 5.37	8.77 \pm 6.95
Muscle	0.77 \pm 0.25	1.16 \pm 0.52

groups received epinephrine. One hour after the epinephrine injection, glycogen content of liver and of skeletal muscle was determined. In the liver, the glycogen concentration was very much lower in those animals previously injected with α -TPh. While the mean value of muscle glycogen was higher in rats injected with α -TPh, the scatter of the data was sufficiently great to deprive them of statistical significance. These experiments are being continued with some improvement in technique. In two series of four rats each, the sharp difference between glycogen concentrations has persisted in the liver, while the difference between the means of muscle glycogen concentrations (2.29 mg. glycogen/g. of muscle after α -TPh plus epinephrine; 1.42 mg. glycogen/g. of muscle after epinephrine alone) remains without statistical significance.

It is possible, however, that a physiological relationship may be obscured here, owing to the wide variation in muscle glycogen content of normal rats. For example, Horvath,⁴ employing a painstaking technique in 409 rats, found concentration of muscle glycogen to vary widely from traces to nearly 1 per cent.

However, with the understanding that the differences in glycogen content in *muscle* may not be real, let it be assumed for a moment that the data are valid. The difference between the means of the glycogen concentrations in liver was 23.3 minus 8.8 = 14.5 mg. of glycogen per gram of liver. In a

200 g. rat, assuming the liver to be 3.8 per cent of the body weight, this difference represents 110 mg. of glycogen per liver. If our hypothesis is correct and the velocity of liver glycogenolysis was unaffected by α -TPh, the difference is the result of resynthesis of 110 mg. of glycogen from muscle lactic acid in rats receiving epinephrine alone. The difference between the means of glycogen concentration in muscle, as determined by the revised technique currently employed, was $2.29 \text{ minus } 1.42 = 0.87 \text{ mg. of glycogen per gram of muscle}$. Again, in a 200 g. rat, assuming muscle to be 45 per cent of body weight, this difference represents 78.4 mg. of glycogen per total muscle mass; or, 78.4 mg. of glycogen was consumed in muscle after epinephrine alone, in excess of that consumed after α -TPh plus epinephrine. This amount is of the same order as the more reliable calculation derived from determinations of liver glycogen concentrations. There is no reason, therefore, for abandoning the working hypothesis that α -TPh interferes with the breakdown of muscle glycogen to glucose but does not affect hepatic glycogenolysis. Obviously, the hypothesis must be challenged by direct test in the muscle phosphorylase system.

During the course of manometric experiments, it was observed that diaphragms from rats injected with α -TPh were immobile when placed in Krebs-Ringer-phosphate solution, whereas normal diaphragms usually displayed waves of contraction for some minutes. This led to beginning a systematic study of the influence of injected α -TPh on a nerve-muscle preparation of the intact rat. The technique employed has been described previously.⁵

FIGURE 3 presents the effect of α -TPh on the electromyogram of the rat

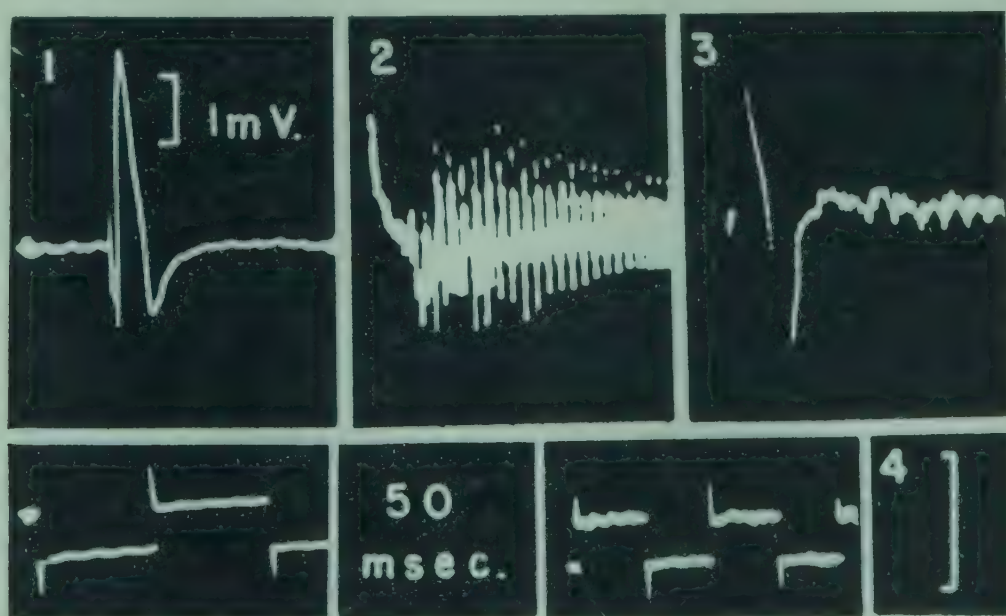


FIGURE 3. Electromyograms recorded from *triceps surae* of intact curarized rat. Stimulus, direct, single shock.

1. Normal electromyogram.
2. Electromyogram in myotonia produced by 2,4-D.
3. Suppression of myotonia by injected α -TPh.
4. Voltage calibration for frames 2 and 3; $50 \mu \text{V}$; time scale, 50 msec.; left applies to frame 1; right applies to frames 2 and 3.

made myotonic by administration of 2,4-dichlorophenoxyacetate.⁵ In the upper left-hand corner is the normal spike potential in response to a single shock. The center panel is a reproduction of the myotonic response. A single shock evokes a long burst of repetitive discharges following the spike

potential. In the upper right-hand corner is illustrated suppression of myotonia by administration of α -TPh.

In the normal rat, a relatively small dose of α -TPh produced an increase in twitch tension, developed isometrically, followed by a fall in twitch tension. With increasing doses of α -TPh, the rise in twitch tension became less prominent and the fall more persistent. With a very large dose, the only effect was a marked decrease in twitch tension (FIGURE 4).

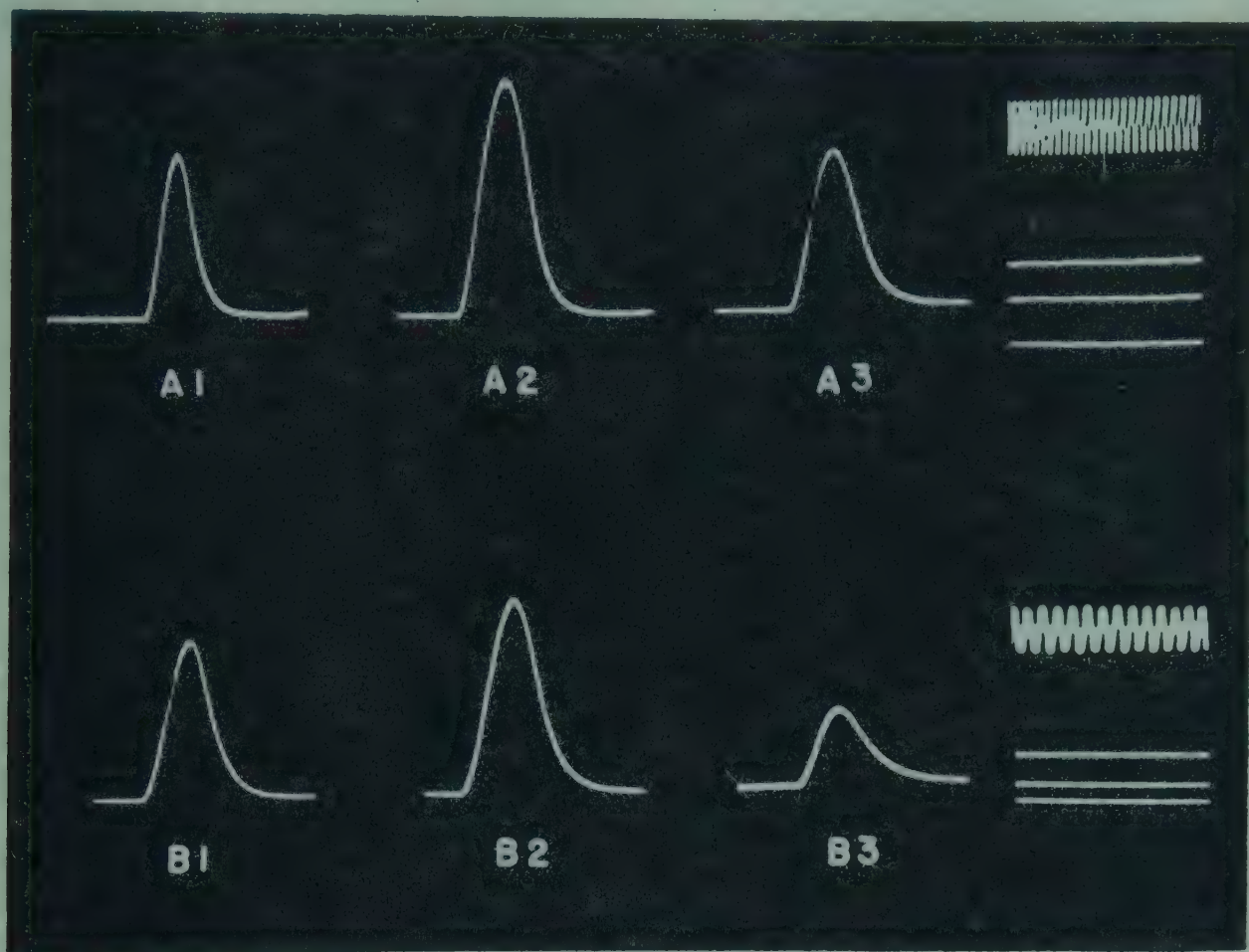


FIGURE 4. Isometric myograms recorded from *triceps surae* of intact rat.⁵ Stimulus indirect through distal segment of cut sciatic nerve, single supramaximal shock once every eight seconds.

A1. Myogram before injection of α -TPh.

A2. 78 minutes after injection of α -TPh, 0.25 g./K.

A3. 6 hours after injection of α -TPh, 0.25 g./K.

B1. Myogram before injection of α -TPh.

B2. 47 minutes after injection of α -TPh, 0.5 g./K.

B3. 3.5 hours after injection of α -TPh, 0.5 g./K.

Calibrations: time scale—100 c.p.s.; tension scale—bottom line = zero tension, middle line = resting tension, and upper line = resting tension + 100 g.

These effects of α -TPh on the nerve-muscle preparation may be manifestations of its interference with explosive energy transfer. If α -TPh does depress muscle glycogenolysis, obliteration of myotonia and reduction in twitch tension may be in consequence of this impairment. Information secured to date, however, offers no explanation for the facilitation of contraction noted in normal rats after smaller doses of α -TPh.

In summary, then, the effects of administered α -TPh are to be distinguished from many of those it exerts *in vitro*. It is suggested that certain of these latter phenomena are manifestations of non-specific (detergent) surface activity. Administered to the rat, α -TPh produced muscular weakness, depression of basal oxygen consumption, and depression of endogenous respiration in skeletal muscle but not in liver or in brain. Some evidence is in harmony with the hypothesis that these effects are in consequence of impaired glycogen phosphorolysis, but proof is incomplete. Administered

α -TPh modifies muscle contractility and excitability in a manner which may reflect altered muscle metabolism induced by that agent.

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Discussion of the Paper

DR. WILBUR H. MILLER (*Chemotherapy Division, Stamford Research Laboratories, American Cyanamid Company, Stamford, Connecticut*): I should like to caution against the use of the word detergent, with its implication of non-specific effects. I believe a better term for such compounds in biological phenomena is "surface-active," which implies only that in the molecule there is some balance between hydrophilic and hydrophobic groups. This would tend against unwarranted generalizations and result more in each compound being judged for its own effects. The question of a specific effect of compounds upon a given enzyme system should be judged, I think, relative to the effect of compounds on *several* enzyme systems. Obviously, compounds with a similar balance of reactive groups, but otherwise not closely related, may also exert the same effect on enzyme systems to varying degrees. In our paper,¹ we have discussed the influence on surface activity which we thought changes in the number of methyl groups in the tocopherol molecule might have. We said that such changes might affect the hydrophilic group relatively about the same, whether it was a phosphate ester or a hydroxyl. Because the phosphate might be considerably more reactive, it would likely thereby be less specific. In an attempt to relate effects observed in a single system with only α -tocopheryl phosphate *in vitro* to vitamin E action *in vivo*, I would agree that considerable caution should be exercised. I believe equal caution should be exercised in labeling such an effect "non-specific" or "detergent" merely because a compound like lauryl sulfate exerts a similar effect to a greater or lesser degree in the same system. Possibly, before any correlation with biological activity is justified in the tocopherol series, *in vitro* work with more than one phosphate ester (or even more than one tocopherol) should be carried out.

DR. P. D. BOYER (*Division of Biochemistry, University of Minnesota, St. Paul, Minn.*): It is of interest, as Dr. Miller has pointed out, that both

tocopherol and tocopheryl phosphate have hydrophilic and hydrophobic portions. However, the properties conferred by the neutral hydroxyl group are much different from those due to the presence of the anionic phosphate group. In reference to the interaction with serum albumin mentioned earlier, the extent of combination of anions with non-polar groups is much greater than the combination of similar compounds without the anionic group but with neutral solubilizing groups. Interpretation of the effects of the phosphate ester as representative of the effects of the free tocopherol should thus be made with caution.

CHEMICAL AND BIOLOGICAL STUDIES RELATED TO THE METABOLIC FUNCTION OF VITAMIN E*

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Three different phases of current research activities on the metabolic function of vitamin E are included in this report. These are: (1) the isolation and tentative characterization of a reversible oxidation product of tocopherol; (2) the effect of α -tocopheryl phosphate and other compounds of similar properties on the succinoxidase system; and (3) a re-evaluation of the biological activity of compounds related to vitamin E.

A Reversible Oxidation Product of Tocopherol. Evidence for the existence of a reversible oxidation product of tocopherol was first obtained in this laboratory two years ago in spectrophotometric studies of the oxidation of α -tocopherol. In these experiments, tocopherol was oxidized by two equivalents of ferric iron in the presence of excess 2, 2'-bipyridine to react with the ferrous iron produced. When the oxidized tocopherol was reduced by ascorbic acid immediately following the completion of the oxidation, the product showed the same ultraviolet absorption curve as α -tocopherol. If the reduction was carried out at subsequent intervals up to 20 hours, the presence of increasing amounts of the irreversible oxidation product, tocopheryl quinone, was evident. These observations suggested that the tocopherol was first converted to a primary oxidation product which could be readily reduced to the original tocopherol or converted irreversibly to the tocopheryl quinone. Experiments were then undertaken to find if such a product existed.

Further experiments showed that by careful oxidation of the tocopherol at $-5^{\circ}\text{C}.$, followed by extraction and chromatographic separation on alumina, the primary oxidation product could be obtained as a colorless oil. Elementary analyses of the product indicated that it has the composition $\text{C}_{29}\text{H}_{50}\text{O}_3$. Thus it is isomeric with the tocopheryl quinone and differs from tocopherol only in the presence of an additional oxygen atom. The relationships existing between tocopherol and its oxidation products are summarized in FIGURE 1. α -Tocopherol may be converted by a reversible, bivalent oxidation to a primary oxidation product. The semiquinone radical of tocopherol¹ is probably an intermediate in the formation of the product. Upon exposure to very dilute acids, the primary oxidation product is converted irreversibly to tocopheryl quinone, the first oxidation product of tocopherol previously attainable.² The tocopheryl quinone is reduced much less readily than the primary oxidation product, and the reduction gives rise to tocopheryl hydroquinone.²

Interest in the primary oxidation product was heightened by the observation that it was biologically active. When fed orally, the product had $\frac{1}{30}$ of the activity of d,l- α -tocopherol, as measured by the conventional rat assay. When administered by intraperitoneal injection as a suspen-

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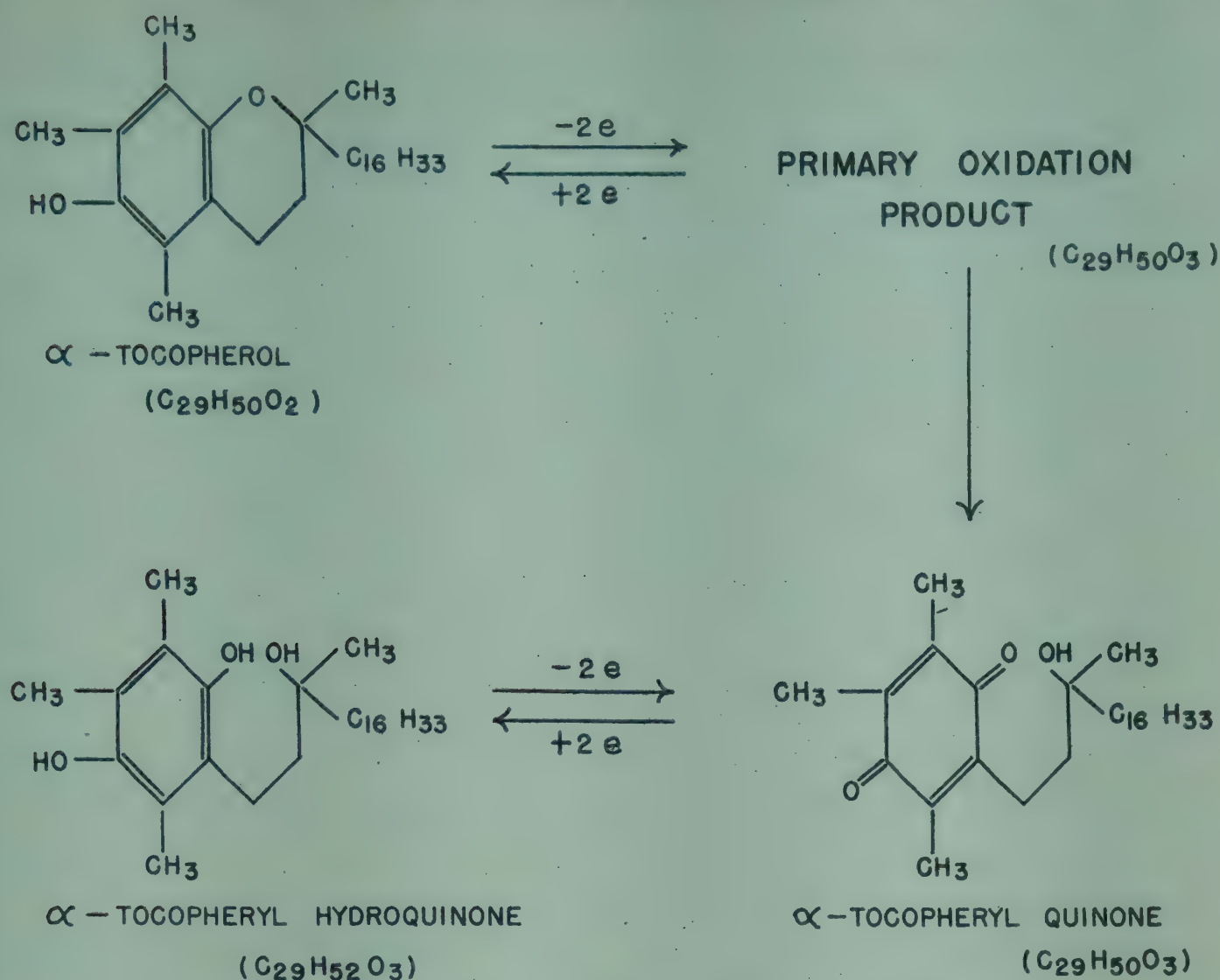


FIGURE 1. The relationships between tocopherol and its oxidation products.

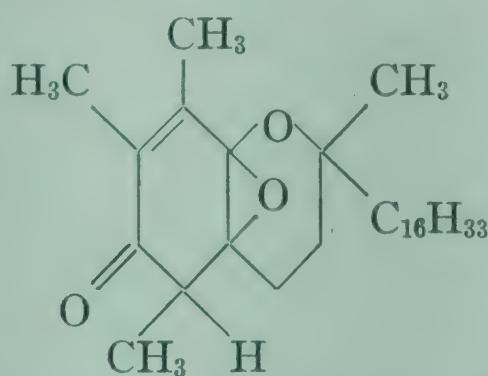
sion with "Tween 20," the product had $\frac{1}{3}$ of the activity of d,l- α -tocopherol. The lower relative activity by oral administration may be related to the ease with which the product forms the biologically inactive tocopheryl quinone.

Further study of the compound indicated that it had an unusual structure. Because of this and the lability of the molecule, it has been possible to assign only a probable structure to the product. Some of the principal observations which aid in assignment of a structure will be briefly summarized. The absence of a hydroxyl group is indicated by failure of the compound to react with acid anhydrides or chlorides, or with isocyanates, diazomethane, or ketene. These observations and the ease with which the product reverts to tocopherol suggest strongly that the hetero ring is intact. The extra oxygen must thus be associated with the carbon ring. Additional evidence as to the structure may be deduced from the ultra-violet absorption spectra. The primary oxidation product shows a strong absorption at $237\text{ m}\mu$ in iso-octane ($\epsilon_M = 11.9 \times 10^3$), in contrast to the bicuspid peak at $260\text{--}267\text{ m}\mu$ ($\epsilon_M = 17 \times 10^3$) shown by tocopheryl quinone. Of the limited number of possible structures, that of an α - β unsaturated ketone³ most readily explains the strong absorption at $237\text{ m}\mu$. Direct reaction with carbonyl reagents has not yet been feasible, because of the lability of the compound to acid and the presence of an active oxygen and because of the methyl groups adjacent to the carbonyl.

Further support for these structural relationships may be drawn from the infra-red absorption spectra. The very characteristic absorption of

the hydroxyl group in the 3 micron region is lacking in the primary oxidation product, but present in the spectra of α -tocopherol and α -tocopheryl quinone. The quinone and the primary oxidation show absorption at 6.1 microns, which is characteristic of the carbonyl group. As expected, this absorption is not shown by α -tocopherol.

The number of possible formulas for the primary oxidation product may be limited by several considerations. The formulation as an oxonium compound, such as that suggested for the unstable intermediate noted in the oxidation of α -tocopherol at the dropping mercury electrode,⁴ is ruled out because of the ultraviolet absorption spectra and other properties of the molecule. The primary oxidation product will liberate iodine from sodium iodide in acetic anhydride, and any structure must account for this oxidizing property. An attractive formula to explain the presence of an active oxygen is that of a transannular peroxide, where the peroxide is present in a ring structure. However, evaluation of the ultraviolet absorption spectra of such compounds demonstrates that they will not account for the strong absorption in the 240 m μ region. From these and other considerations, it is concluded that one of the oxygens associated with the carbon ring must be present as a carbonyl group and the other in a ring structure. The presence of a four-membered ring is not likely, because of the relative stability of such rings. Some three-membered epoxide structures have been found to contain active oxygen.⁵ This, together with the lability to acid and other considerations, suggests that the extra oxygen is present in a three-membered ring. The location of the ring is questionable. The ease of conversion to the quinone suggests that it is present as indicated in the accompanying formula or as the corresponding isomer with the epoxy group in the 8,9 position.



2,5,7,8-tetramethyl-2(4,8,12-trimethyl-tridecyl)-
9,10-epoxy-6(5H)-chromanone.

Primary oxidation products have also been obtained from β , γ , and δ -tocopherols, but with increasing difficulty. These products show absorption maxima approximately 10 m μ lower than the product from α -tocopherol. Also, consonant with the postulated structure, similar crystalline but irreversible oxidation products have been obtained from ethers of durohydroquinone.

Action of Tocopherylphosphate on Succinoxidase. Studies on the action of tocopheryl phosphate on enzyme systems in this laboratory have been concerned chiefly with the succinoxidase system. Tocopheryl phosphate is an anion with a large non-polar group and would be expected to show

properties similar to other such anions, for example, the ability to act as a detergent and to combine with and denature protein molecules. Thus, effects of α -tocopheryl phosphate may not reflect the biological action of vitamin E. Earlier in this monograph, Dr. Ames presented data supporting the interpretation that the *in vitro* effects of α , γ , and δ -tocopheryl phosphates are not correlated with the biological activity of the tocopherols.

The non-specificity of the inhibition of the succinoxidase system by α -tocopheryl phosphate is indicated by the similar effect produced by other anions with large non-polar groups. A comparison of the effects of α -tocopheryl phosphate and dodecyl sulfate on the O_2 uptake of the complete succinoxidase system showed that both substances have similar pronounced inhibitory effects. A further similarity of the action of α -tocopheryl phosphate and dodecyl sulfate is that the inhibitory action of both substances can be prevented by other proteins such as serum albumin, which combine non-specifically with the inhibiting agents. In addition, a variety of other surface-active agents have been tested in this laboratory and elsewhere, and it may be generalized that the succinoxidase system of tissue homogenates is readily inhibited by surface-active agents. The inhibition of a variety of enzyme systems by both α -tocopheryl phosphate and other similar anions is further argument that such inhibition does not represent a biological function of tocopherol. Although interpretations of the *in vitro* or *in vivo* effects of α -tocopheryl phosphate should be made with caution, the results do not preclude the possibility that the phosphate ester might have some biological function.

Other experiments have been conducted which give additional information concerning the manner in which α -tocopheryl phosphate inhibits the succinoxidase system. Several experimental results demonstrate that the accumulation of oxalacetate, arising through a protective action of α -tocopheryl phosphate on cozymase, is not the major factor in the inhibition. For example, the addition of 1.9×10^{-4} M α -tocopheryl phosphate at the ten-minute interval to an already functioning liver succinoxidase system resulted in a marked inhibition. Since the system had an active oxygen uptake prior to the tocopheryl phosphate addition, it is evident that insufficient oxalacetate was present for pronounced inhibition during this first period. Protection of any remaining cozymase by addition of α -tocopheryl phosphate should not increase the rate of the cozymase-catalyzed oxalacetate production. Thus, the results suggest that other mechanisms must be operating. More direct experimental evidence that oxalacetate accumulation is not a major factor comes from experiments with methylene blue and heart homogenates, which demonstrate that, under conditions where the succinoxidase system is completely inhibited, the dehydrogenase component is still active. Since oxalacetate is a competitive inhibitor of the dehydrogenase,⁶ the principal inhibitory effect could not be due to the presence of oxalacetate.

Some data in the literature indicate that tocopheryl phosphate has an effect on components of the cytochrome system. We have noted that α -

tocopheryl phosphate (2.5×10^{-4} M) and dodecyl sulfate (9.4×10^{-4} M) cause a marked reduction in the maxima of the absorption spectrum of reduced cytochrome c (0.96×10^{-4} M). In view of the rather striking effect on the absorption spectrum of cytochrome c, manometric studies were made to ascertain if this might offer an explanation for the inhibition of the succinoxidase system. However, it was found that addition of a fresh supply of cytochrome c to heart homogenate preparations partially inhibited by α -tocopheryl phosphate did not result in any increase in oxygen uptake. Some reactivation would have been expected if cytochrome c were the limiting component.

In other experiments, it was demonstrated that cytochrome oxidase was still potentially active in succinoxidase preparations completely inactivated by α -tocopheryl phosphate. In these experiments, the succinoxidase activity was measured in the usual manner, then the cytochrome oxidase was determined by addition of ascorbate from the side arm. This gave a reduction of the cytochrome c which was independent of the action of succinic dehydrogenase. The results showed that levels of α -tocopheryl phosphate which completely inhibited the succinoxidase system only partially inhibited the cytochrome oxidase present.

Attention was thus directed to the succinic dehydrogenase component of the succinoxidase system. This component may be studied separately by the use of methylene blue as a hydrogen acceptor under appropriate conditions. Experiments were conducted in which methylene blue additions were made to rat heart succinoxidase preparations completely inhibited by α -tocopheryl phosphate (4.75×10^{-4} M), dodecyl sulfate (4.75×10^{-4} M), or cyanide (4.7×10^{-3} M). Addition of methylene blue (1.3 or 6.3×10^{-3} M) at the ten-minute interval resulted in a rapid oxygen uptake in the presence of cyanide. This gave a measure of the potential activity of the system with methylene blue. Addition of similar levels of methylene blue to the enzyme system inhibited by α -tocopheryl phosphate or dodecyl sulfate resulted in a partial reactivation. Thus the dehydrogenase component was active in the presence of these inhibitors. If the methylene blue was added prior to the α -tocopheryl phosphate, the activity obtained was nearly equal to that of the control.

These results lead to the conclusion that, in the succinoxidase system completely inhibited by α -tocopheryl phosphate, the cytochrome oxidase and succinic dehydrogenase components are potentially partially or completely active. Two interpretations may be offered:

(1) The α -tocopheryl phosphate in some manner blocks the action of a component coupling the dehydrogenase and cytochrome c. A number of investigators have prepared succinic dehydrogenase that will not react with cytochrome c, and have presented evidence for a factor linking the dehydrogenase with cytochrome c.

(2) α -Tocopheryl phosphate, when absorbed on the enzyme surface, may prevent association with the cytochrome c, but not with the much smaller methylene blue molecule.

Activity of Compounds Related to Tocopherol. A variety of compounds

with widely diverse properties and structures have been reported to possess vitamin E activity.⁷ This has made difficult any correlation of structure with biological function. The formation of an easily reversible oxidation product, as described in the first portion of this paper, is apparently a property peculiar to compounds closely related to the tocopherols. If the formation of such a reversible oxidation product has a biological function, then more exacting requirements for vitamin E activity should exist. A re-evaluation has been made of the biological activity of eight representative compounds, by use of a rat assay essentially as described by Mason.⁸ The compounds tested were 3-carbethoxy-5,7,8-trimethyl-6-hydroxycoumarin, 2,2,5,7,8-pentamethyl-6-hydroxychroman, 2,2-diethylchroman, 2-methylchroman, duroquinone, durohydroquinone, durohydroquinone mono-n-cetyl ether, and durohydroquinone-mono-n-dodecyl ether. Bioassays with rats, using dosage levels comparable to those previously reported active, showed all eight compounds to be devoid of biological activity. One plausible explanation of the discrepancy of these results and those reported earlier may be the occurrence of "first-litter fertility" frequently noted as a complicating factor in the earlier bioassays. In trials concurrent with these experiments, none of 27 negative control animals with established implants developed live young, and the minimum fertility dose of d,l- α -tocopherol ranged from 0.7 to 1.0 mg. The inactivity of the compounds tested casts doubt on the validity of results of earlier assays with these and other compounds, and allows the conclusion that the requirements for vitamin E activity are more specific than heretofore recognized.

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Discussion of the Paper

DR. L. MICHAELIS (*Rockefeller Institute for Medical Research*): It is possible that Dr. Boyer's epoxy compound is the second step in the oxidation of tocopherol, the first step being the formation of the semiquinone radical, recently described (MICHAELIS & WOLLMAN. *Science* **109**: 313, 1949). When tocopherol, dissolved in a mixture of alcohol, ether and pentane, is cooled to the temperature of liquid air and is irradiated with ultra-violet light, it develops an orange-red color with characteristic absorption bands. At the low temperature, this color is stable even after radiation is stopped, but fades out when the frozen solution melts. The free radical of tocopherol is not merely a product of paper chemistry. Tocopherol is hereby placed

in the class of reversible oxidation-reduction systems, of which there are analogous examples among other vitamins.

DR. P. D. BOYER: The observations by Dr. Michaelis on the semiquinone of tocopherol are, as he pointed out, complementary to and not in conflict with the formation of the primary oxidation product we isolated and which was formed by a two electron transfer. The students in my enzyme course at the University of Minnesota would, I am sure, confirm that I am in agreement with the valuable concepts of univalent steps in oxidation as advanced by Dr. Michaelis. Indeed, in view of what is known about similar oxidations, it would be surprising if the free radical were not an intermediate in the formation of the isolable primary oxidation product.

In relation to the source of the extra oxygen in the oxidation product, the product was formed in ethanol solutions containing some water, and thus water or hydroxyl ions provide the probable source of oxygen.

RELATIONSHIP OF VITAMIN E DEFICIENCY TO TISSUE PEROXIDES

By Henrik Dam

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To produce exudative diathesis and encephalomalacia in chicks, as well as the brown coloration of adipose tissue in rats and chicks, highly unsaturated, easily oxidizable fatty acids must be ingested and vitamin E must be absent, or present only in traces in the diet. Diets in which the cod-liver oil is made thoroughly rancid (as measured by the lowering of the iodine value to 60–70) while in contact with the other food ingredients do not produce these symptoms. If the unsaturated fatty acids are oxidized before they are consumed, they have no effect, nor are ingested fat peroxides deposited in the body.³

It seems almost certain that unsaturated fatty acids must be present in the affected tissue. From the work of Sinclair,¹⁰ it is known that ingestion of highly unsaturated fatty acids leads to an increase of the iodine value not only of the depot fat, but also of the tissue phospholipids. It is easy to show that this increase is about the same whether vitamin E is present or not. Therefore, the most likely explanation of the symptoms is that the tissue is affected by some process which the highly unsaturated fatty acids undergo in the cells when vitamin E is lacking.

Since vitamin E is an antioxidant and prolongs the induction period of unsaturated fatty acids, an observation which was made by Mattill and coworkers even before vitamin E was isolated, it is reasonable to believe that the process in question has to do with the oxidation of these fatty acids in the cells.

In order to throw some light on this mechanism, we first attempted¹ to determine peroxides in the depot fat of vitamin E-deficient chicks and rats. The chicks were reared on the then usual exudate-producing diet containing 5 per cent cod-liver oil. Some of the chicks were given the same diet, plus a certain amount of α - or γ -tocopherol acetate. The chemical method used for the determination of peroxides was an adaptation of King, Roschen, and Irwin's iodometric method,⁵ which probably gives figures that are slightly low because part of the iodine set free by the action of peroxides on potassium iodide may be taken up by the double bonds of the unoxidized fatty acids.

These experiments showed that peroxides may be detected in the chick body fat at about the time when exudate is present and that, in most cases of brown discoloration of the fat, the peroxide value has increased. When 2.5 mg. per cent of α -tocopherol acetate were present in the diet, peroxide values were zero and the symptoms were absent.

There are, *a priori*, several ways of explaining the relation between the beginning of peroxidation in the tissue and the effect on the capillaries. Perhaps the oxidation products themselves damage the capillaries, so that hemorrhage and exudation occur. Perhaps the small amount of vitamin

E originally present in the tissue is nearly used up at the time when peroxidation begins, and the lowering of vitamin E in the tissue causes the symptoms. The disappearance of vitamin E, in the presence of highly unsaturated fatty acids, is linked up with its antioxygenic activity.

Before we venture further into the theory of this process, I wish to report a very simple experiment which was carried out in order to determine whether cod-liver oil undergoing rancidification in the body will give rise to exudation, as happens when oil of turpentine is introduced intraperitoneally, a method commonly used for obtaining exudate fluid from animals. Oil of turpentine, by the way, also forms peroxides.

A series of chicks receiving a normal commercial diet were injected subcutaneously with 0.3 ml. of cod-liver oil. They were inspected daily for exudation but none was found. At intervals of one or more days, the site of injection was opened and as much of the remaining oil as possible was taken out for peroxide determination. Considerable peroxidation was found, with a maximum of 200–300 milliequivalents per kg. fat at about the fourth to the eighth day after the injection. At the same time, a yellow-brown color developed in the oil-drenched tissue.

In another series of such experiments, α -tocopherol was added to the cod-liver oil before injection. A rather large amount of free tocopherol was used, viz.: 2.4 mg. in 0.3 ml. cod-liver oil. This did not alter the results. The peroxidation and yellow-brown color developed to the same extent and just as rapidly as when cod-liver oil without tocopherol was injected. This is in agreement with what is found when cod-liver oil is left in contact with air at body temperature with or without tocopherol and demonstrates very strikingly that tocopherol is quickly consumed under such conditions.

In feeding experiments, when tocopherol and cod-liver oil are given daily in the food, conditions are different. Then, the resulting concentration of highly unsaturated fatty acids in the fat tissue is lower than it is in cod-liver oil exposed to air, because the supply of tocopherol is renewed every day to replace that which is lost by oxidation. This could be the explanation as to why tocopherol is an effective antioxidant in the fat tissue in the body but not in the injected oil depot or *in vitro*.

Turning now to the rat experiments, in which a high level of cod-liver oil (20 per cent of the diet) without tocopherol was fed, we found that a brown discoloration of the adipose tissue was preceded and accompanied by very marked peroxidation, peroxide values as high as 30–70. The figures were especially high when nursing rats, together with their mothers, were given the diet from birth, indicating that some of the highly unsaturated fatty acids pass through the milk from mother to young. Exclusion of cod-liver oil or the presence of vitamin E in the diet protected against discoloration and peroxidation.¹

These experiments must be interpreted to mean that the brown discoloration of the adipose tissue is caused directly by peroxidation of the highly unsaturated fatty acids in the adipose tissue in the absence of a daily supply of tocopherol. The color is probably not due to the peroxides

themselves but to other products formed from them in the further course of rancidification.

Why there are two components of the pigment, one fat-soluble, the other fat-insoluble, is not difficult to explain. The former probably represents less polymerized oxidation products of the fatty acids, while the latter components may consist of polymers of the oxidized fatty acids so highly polymerized that they are insoluble; or, it might consist of the combination of such polymerization products with protein. This component may be extracted from the tissue with dilute alkali, but even after this treatment it cannot be brought into solution in fat solvents. Moore and Wang⁹ set forth another explanation, *viz.*: that the brown acid-fast pigment is formed by oxidation of proteins.

Martin and Moore^{6,7,8} found that vitamin E deficiency in rats led to brown coloration of the uterus. In our experiments some coloration of the uterus appeared even without cod-liver oil in the diet. It is possible that uterine muscle has a tendency to conserve or attract small amounts of unsaturated fatty acids which by oxidation give rise to the pigment.

In order to look further into the development of the pigment, and also into the mechanism whereby vitamin E may be destroyed in the body under the influence of the highly unsaturated fatty acids, we must consider briefly some of the recent theories of the process of auto-oxidation of fatty acids.

According to E. H. Farmer and his coworkers² (based upon *in vitro* experiments), the oxidation of fatty acids by molecular oxygen is catalyzed by the formation of a free radical in the initial stage of the process. This free radical initiates a chain reaction. The action of tocopherol or other antioxidants is to break the chains by removing the free radicals. The antioxidant is thereby destroyed. When all the tocopherol is oxidized, there is nothing to slow down the peroxidation process. Possibly, it is the free radical which produces the damage to the capillaries in chicks and thereby gives rise to hemorrhage and exudation in the adipose tissue. It is not known why exudation does not appear in rats.

If the reaction chains are not broken, hydroperoxides, some of which have been isolated by Farmer *et al.*, will, in the course of the rancidification, be converted into keto and hydroxy compounds and further oxidized. Some of the products may become polymerized. These substances are brown and show a greenish-yellow fluorescence when exposed to ultra-violet light.

A chemical method for the determination of peroxides in fat which is more sensitive than the usual iodometric procedure has been worked out in the authors' laboratory and adapted to the histochemical demonstration of fat peroxides.⁴

This method is based on the oxidation of leuco-2,6-dichlorophenolindophenol by peroxide. The leuco-dye is dissolved in n-butanol with 5 per cent glacial acetic acid to make a 1 to 2 per cent solution. Of this, 0.1 to 0.2 ml. is mixed with 3 ml. xylene containing the oil to be examined. The

mixture is then heated 10 minutes on a water-bath at 70°C. Peroxides in the oil give rise to a red color which can be measured in the Beckman spectrophotometer at 520 m μ ; 0.01 milliequivalent of peroxide per kilogram oil can be determined. The method is, thus, 10 to 100 times as sensitive as the iodometric method of King, Roschen, and Irwin.⁵

The method can be applied histochemically to frozen sections if a catalyst is used so as to avoid heating. Hemin is suitable for this purpose. Two solutions are prepared: (1) (stable) 20 mg. hemin is dissolved in a mixture of 5 ml. pyridine and 10 ml. glacial acetic acid; (2) (must be prepared fresh) 25 mg. of leuco-2,6-dichlorophenolindophenol dissolved in 3.5 ml. of absolute alcohol and 5 ml. of distilled water. 0.74 ml. of 1 is mixed with the whole amount of 2. When the mixture of the two solutions is applied to the frozen sections, the red color develops almost immediately in the places where peroxides are present. The excess of staining solution is removed by rinsing with distilled water. The fact that 2,6-dichlorophenolindophenol is fat-soluble in acid media is the basis of the method. Leuco-dyes which form non-fat-soluble dyes on oxidation will not stain the peroxidized fat in the tissue.

This method has been applied to the study of the relationship between peroxidation and the yellow-brown coloration of the adipose tissue in vitamin E-deficient rats. The yellow-brown acid-fast pigment found in the fat cells at different stages of development does not always exhibit peroxides. However, peroxides always seem to be present in the fat depots when the formation of pigment begins, thus suggesting that the acid-fast pigment represents changes of the unsaturated fatty acids beyond the peroxide stage.

When the same staining method is applied to brain tissue, some difficulty is encountered, in part due to the presence of reducing substances which interfere with the reaction between the leuco-dye and the peroxides. Further, oxidized lecithin does not give the reaction with leuco-dyes, perhaps because in lecithin the oxidation of the fatty acids immediately proceeds beyond the peroxide stage. Peroxidation has, therefore, not yet been demonstrated in brains exhibiting encephalomalacia.

Summary

Exudates in chicks and brown coloration of the fat tissue in rats and chicks are caused by the feeding of vitamin E-deficient diets containing a sufficient amount of highly unsaturated fatty acids. The same applies to encephalomalacia in chicks and the depigmentation of the incisors in rats.

The development of exudates in the adipose tissue of chicks coincides approximately with the time when peroxides can be demonstrated in the depot fat. When brown coloration of the fat tissue occurs, the peroxide value of the fat is increased.

A histochemical method for the demonstration of peroxides was applied to frozen sections of the brown adipose tissue of rats. Peroxidation of the fat always seems to precede and be a necessary stage in the formation of the brown pigment, but the pigment itself does not always show peroxida-

tion. It is likely, therefore, that the pigment represents further stages of oxidation and polymerization of highly unsaturated fatty acids beyond the peroxide stage.

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PROTECTIVE ACTIONS OF VITAMIN E IN CONDITIONS OF
METABOLIC STRESS

ADDRESS ON VITAMIN E

*By K. Hickman**Rochester, N. Y.*

Vitamin E is unique in being, to the best of my knowledge, the only vitamin to have commanded two international conventions. That this has come about is due to the perseverance of a score of scientists who, over a score of years, have placed their faith in vitamin E, believing that it would one day win a central place in the science of nutrition. In the vanguard, are Drs. Mattill, Mason, Evan Shute, and Pappenheimer and the synthetic drug houses; waging the battle today, Drs. Harris, Hove, Quaife, Ames, Boyer, Burgess—but to mention names is inevitably to omit names, and this is unfair. The protagonists of vitamin E can console themselves that in spite of a bad press and rather vehement medical incredulity, as well as lack of notice or acceptance by research councils and nutrition departments, vitamin E has become accepted by the public as a valuable supplement to diet.

The question we have asked so often is—how does vitamin E function? We sometimes forget that the E-vitamins and their derivatives are highly poly-functional, perhaps more so than any vitamin, and we already know many of their clinical properties. Vitamin E and cancer are in the same situation in that by continually harping on what we don't know, we often fail to apply what we do know. Today, we are hunting for a vitamin E enzymic function. Almost, I wish we would not find it, because we are so likely to say, well, well, that's *that*—and forget the rest or fail to look for more.

There is a paradox, probably linked with the enzymic function of vitamin E, that I think should be in the foreground of our attention. The basic need for vitamin E is in adolescence, when the new tissue that is being manufactured must be endowed with α -tocopherol-of-constitution. Maturity has no such basic need; the construction period is over. Yet, in common experience, we find the situation reversed. The new-born infant flourishes and grows on a store of α -tocopherol so small, relatively speaking, that it would throw the adult into acute dystrophy! The aging human, according to current clinical testimony, may require fifty times the usual intake to maintain health. Evidently, the babies' machine for utilizing tocopherol is much more efficient than the older adults'. Are we always clear in our thinking, I wonder, in distinguishing between failure to utilize and failure to obtain vitamin E? At our next conference, it is to be hoped that we shall hear much more about the machinery of utilization, even if this throws the matter right back to the enzyme chemists.

There is an aspect of "co-vitamin E" activity that I should like to mention before closing. Experimental biochemistry uses freely two terms: *in*

vitro and *in vivo*. Have you thought, however, that the fundamental enzyme reactions, which are synonymous with living, involve in the microsecond of a molecular exchange only a very few molecules, perhaps one in a billion of the body's contents? All the other molecules are on the journey to the site of interaction; or their metabolized parts are diffusing away; or they form part of the inactive, supporting structure of the body. Evidently, a third term—*in transitu*—could be used to crystallize our thinking about the vast majority of the contents of living matter.

The molecules *in transitu* are, by definition, not subject to primary biologic processes, but we know they are amenable or vulnerable to uncounted secondary changes or losses. One has only to think of the varying degree of survival of vitamin C or carotene according to chemical environment in the cook-pot, the intestines, the liver, or the blood stream, to realize the importance of the *in transitu* history on the ultimate *in vivo* activity. We believe that vitamin E, when synergized to the optimum in the body, is an important biologic *in transitu* preservative agent both to the metabolites and to the structural parts.

The connection with longevity, as Dr. Kaunitz has emphasized, is inescapable. It seems to me that the chemical factory of the body must have to cope with many of the tiresome practical problems of the organic chemist. The chemist reacts his constituents and claims a 70 per cent or 90 per cent yield and concedes a loss of 5 per cent or 15 per cent in side reactions. But he generally finds, also, a modicum of highly polymerized residue, perhaps less than one per cent, that resists being cleaned from his flask. Surely the over-elaborated fragment, the resin or tar, must accumulate in the cell and the intercellular spaces of the body to a greater or lesser extent according to the perfection of *in transitu* protection. Are not the pigments of Mason and Dam magnificent examples of tars left in the body reactor because of deficient chemical management? Is not the accumulation of "dirt" in the form of indiffusible macro-molecules a central phenomenon of the aging organism? Does not vitamin E typify, if it does not wholly contain, the answer to the problem of organic housekeeping and tidiness?

So convinced am I of the interconnection between *in transitu* chemistry, vitamin E, and aging, that I have spent some few moments searching the poets for an appropriate line to leave in your memories. Swinburne's *Atalanta* has provided the quotation and may his spirit pardon the liberties I have taken with the text:

"Seeing that in Death
There is no comfort and none aftergrowth,
Pray thou thy days be long before thou die
And full of E's and Kingdom."

AN EVALUATION OF VITAMIN E DEFICIENCY IN THE YOUNG ADULT RAT*

By Cosmo G. Mackenzie

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At the time of the International Conference on vitamin E held ten years ago, the only species known with certainty to require this factor was the rat. Moreover, even the rat did not appear to need the vitamin for the maintenance of life, except for the brief period encompassing its foetal development and infancy. On the day before the first Conference on E convened, it was reported in *Science* that the vitamin is essential for the survival of the rabbit.^{1,2,3} Shortly thereafter, it was shown that E performs a vital role in the nutrition of the guinea pig,⁴ chicken,^{5, 6} duck,⁷ and other species. In addition to greatly increasing the stature of E among the vitamins, these discoveries revealed the fact that E deficiency is responsible for such dissimilar pathological reactions as nutritional muscular dystrophy in herbivora,⁸ and nutritional encephalomalacia in the chicken;⁹ diseases that had been described eight years earlier and attributed to the absence of some still unknown factor or factors.

The qualitative differences in the morphological response evoked by E deficiency in various animals is sufficiently marked to distinguish vitamin E from the other known vitamins. Consequently, our understanding of the cause of this phenomena is essential to an understanding of the biochemical action of vitamin E and to the rational application of vitamin E in human therapy. Of even greater consequence is the light that the solution of this problem will shed on the differences in metabolic mechanisms elaborated by higher animals in the process of evolution, for there can be no doubt that different qualitative or quantitative biochemical reactions underlie the various pathological effects of avitaminosis E.

The rat is a valuable animal in the study of this problem, since it not only differs from many other animals in its response to E deficiency, but it also manifests, within its own lifetime, different reactions to the deprivation of E. Inasmuch as the papers to be presented in this section will describe the impact of E deficiency on young rats exposed to several conditions of metabolic stress, it is perhaps appropriate to review briefly some of the earliest observations on the avitaminosis in the rat and to consider their implications with respect to the mode of action of the vitamin.

When E deficiency develops in the suckling rat, there occurs an extensive degeneration of the skeletal muscles that is usually fatal.^{10, 11} However, if healthy 21 day-old rats are placed on an E-deficient but otherwise well-balanced diet, they develop no striking gross symptoms, other than reproductive failure, until they are 8 to 12 months old. At this time, a

* This paper was prepared as an introduction to this section on Protective Actions of Vitamin E in Conditions of Metabolic Stress. In actual fact, only the last part was used for this purpose, most of the points raised in the first part having been previously introduced into the discussion following papers presented in the previous sections. No attempt has been made to review the current literature. On the contrary, an effort has been made to refer to the contribution that first established a particular fact, in the belief that the original paper frequently contains important subsidiary results and a fresh point of view that are often buried under the rapid accumulation of new contributions, including those from the same laboratory.

progressive paralysis of the hind legs sets in¹² that is accompanied by tremors and incoordination of the fore limbs, neck, and head.¹³ Morphologically, the striated muscles of these old paralyzed rats show degenerative changes resembling and approaching in extent those found in the suckling young.^{14,*} It is perhaps significant that these severe muscle lesions occur at the extremes of the life span of E-deficient rats, but we do not yet know whether the muscle damage in the older animals is due to a heightened susceptibility to E deficiency or to the prolonged deprivation of the vitamin.

Between these two periods in its life, when the animal is growing rapidly and when it is in its prime, only occasional damaged muscle fibers are to be found, about one per two or three low power fields. It is all the more significant, therefore, that severe biochemical lesions are present in the muscles at this time, for the possibility that they are secondary to morphological degeneration is excluded. Thus, in five-month-old E-deficient rats, the maximum isometric tension developed by direct stimulation of the intact gastrocnemius muscle is reduced by 30 per cent.^{15, 16} The creatine content of the muscle is lowered and the water and chloride content are increased.^{15, 16} At the same time, there is a 40 per cent increase in the oxygen consumption of striated muscle.¹⁷

Another symptom which may be observed after approximately 3 months on an E-deficient diet is discoloration of the uterus due to the presence of yellow granules in the smooth muscle cells.^{14, 18} More recently, the widespread distribution of this pigment in the smooth muscle tissue of older animals has been described.¹⁹ It is also found in the lesions of the skeletal muscles and in the sex glands.¹⁴ Moreover, after only two months on the deficient diet, the pigment is found in macrophages, which have transported it from muscle cells to the lymph nodes.¹⁹ The ingestion of large amounts of unsaturated fatty acids, particularly linolenic acid, results in the formation of a similar or identical pigment in the fat of adipose tissue cells,^{20, 21} with a simultaneous increase in the peroxide content of the fat.²² In contrast to this formation of pigment in the young E-deficient rat is the simultaneous loss of the naturally occurring yellow pigment of the maxillary incisors.

While the *in vitro* function of E as an antioxidant has long been applied to the interaction of the tocopheroles with pro-oxidants in the diet and in the gut, there has been, until recently, some hesitancy in extending the antioxidant action of E to living cells. Yet, as early as 1941, it was shown that E not only increases the storage of vitamin A in the liver of the young rat²³ but also exerts a marked protective action *in vivo* on the vitamin A stores of this organ.²⁴ Conversely, at the same time, it was shown in the rabbit that the administration of cod-liver oil, under conditions that precluded its interaction with E in the gut, increased the antidystrophy requirements for the vitamin,²⁵ a finding that parallels the *in vitro* destruction of E by unsaturated fats.

The effects of E deficiency in the young rat outlined above—increased

* In comparing these lesions, allowances must be made for the difference in their rate of development in the suckling and adult animals.

oxygen uptake by the muscles, pigment formation, increased peroxide content of the body fat, and preservation of vitamin A in the liver—all suggest that E is functioning as a mentor or inhibitor in several oxidative reactions, either by limiting the rate of some normally occurring reactions or by preventing the occurrence of detrimental reactions that take place only in its absence. With respect to the increased oxygen consumption of striated muscle tissue, an alternative possibility exists, namely: that vitamin E functions in the muscle not as an antioxidant but as a coenzyme in an endergonic reaction such as the formation of the high energy phosphate bonds of phosphocreatine,² adenosinetriphosphate,³⁰ *etc.* In such an event, vitamin E deficiency might result in a compensatory increase in those oxidative reactions that provide energy for the synthesis of high energy bonds. The demonstration that α -tocopherol can form a semiquinone,²⁶ and, hence, a reversible oxidation-reduction system, is compatible with both the antioxidant and coenzyme theories just set forth, and they are, of course, not mutually exclusive. It appears to the writer, on the basis of our current knowledge of the effects of E deficiency in the rat, that a major role of the vitamin *in vivo* is the control or restraint of one or more oxidative reactions. The question remains as to whether it also plays a direct role in accelerating some endothermic reaction.

The early finding that the *in vitro* antioxidant activity of several tocopherols is inversely proportional to their biological potency need not trouble us, for later work²⁷ indicates that this relationship does not hold at body temperature. In any event, the entire structure of a molecule, and not only the reactive groups, governs its biochemical activity.

Although the tocopherols are fat-soluble substances, their function as inhibitors (or accelerators) in oxidative reactions is not necessarily restricted to lipid metabolism. Many oxidations in the living cell do not occur in a dispersed system, but rather on the surface or in the interstices of complex structures, such as the mitochondria. The latter are composed of lipids, proteins, nucleoproteins, coenzymes, *etc.*²⁸ and contain the enzymes for oxidizing such diverse substrates as fatty acids and the components of the tricarboxylic acid cycle.²⁹ It is quite possible that the tocopherols are associated with these or similar centers of oxidation. Since so little is known concerning the extent to which metabolic reactions are controlled by naturally occurring inhibitors, the determination of the intracellular distribution of E is of prime importance.

How is the hypothesis that vitamin E functions as an inhibitor or mentor of a number of oxidative reactions in the young rat to be reconciled with the fact that it is not essential in these animals for the maintenance of life? This disparity is all the more striking when one considers that the suckling rat dies when it is deficient in vitamin E, as do the adults of many other species. The explanation would appear to lie in one of several possibilities. Perhaps, at about one month of age, there occurs a shift in the rat's metabolism from a series of reactions in which E is essential to a new series of reactions in which E is less vital. Or, alternatively, with the passage of the first month of life, the rat may develop the ability to syn-

thesize, in increased amounts, compounds that partially substitute for the vitamin. A third possibility is that the young rat, or its bacterial flora, acquires the ability to synthesize tocopherols in limited quantities. At the moment, we do not know which of these conditions corresponds to fact. Nevertheless, if vitamin E can fulfill such an important function as an inhibitor or mentor in oxidative reactions, then it should be possible to reveal differences between normal and E-deficient rats—differences as divergent as life and death—by subjecting them to conditions of metabolic stress that are normally foreign to their carefully guarded and complacent laboratory existence. It is with the results of such experiments that the next four papers are concerned. They show, in striking fashion, that under some circumstances vitamin E is indeed essential for the survival of the young adult rat and that the dispensibility or indispensibility of this vitamin for survival is defined, therefore, by the nature of the environment. In consequence, the implications of these experiments with respect to possible uses of vitamin E in medicine are considerable. Finally, they bring new light to bear on the role played by the tocopherols in biochemical systems.

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THE SIGNIFICANCE OF PROTEIN IN VITAMIN E DEFICIENCY

By T. Moore*

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The possibility that vitamin E may be related to protein metabolism has been suggested by several workers. Dam¹ gave groups of weanling rats a diet deficient in vitamin E with or without supplements of tocopheryl acetate, and, at points 1–4 weeks later, in different groups, replaced the usual casein component by additional carbohydrates. In the groups dosed with tocopherol, the mean survival times were invariably greater than in the groups given no vitamin E, while the body weights of the dosed animals fell to lower levels than those of the undosed animals before death occurred. Victor and Pappenheimer² found that rats failed to grow normally when fed upon diets low in protein, containing cod-liver oil, and either with or without the addition of cystine. The administration of α -tocopherol, however, prevented the failure in growth and delayed the appearance of ceroid pigment in the liver.

In adult male rats which were given a diet low in protein, Hove³ found that treatment with α -tocopherol had little effect on the fall in body weight for the first 4 weeks. After this point, however, the animals without the vitamin continued to decline and showed signs of muscular dystrophy, while the dosed animals suffered no further fall in weight and remained in good condition. In this work, it was also found that the dental depigmentation reported by Moore⁴ in rats deficient in vitamin E was more severe when the diet was low in protein than when adequate amounts were given, although dosing with tocopherol ensured normal dental pigmentation irrespective of the protein intake.

In later experiments, Hove and Harris⁵ found that α -tocopherol increased the efficiency of utilization of protein by rats when levels of casein between 6 and 12 per cent were given, but observed no benefit when the casein allowance was either below or above these limits. The tendency to dental depigmentation of the upper incisors caused by protein deficiency was partially prevented by α -tocopherol, and the incidence of stomach ulcers was reduced. In extension of the observations of Schwarz,⁶ xanthine, or an autolysate of yeast protein, appeared to have much the same beneficial effects as tocopherol. Tocopherol was also found to protect rats kept on a diet moderately low in protein against injury caused by injections of carbon tetrachloride.⁷ Theophylline, and to a lesser degree xanthine and guanine, were also protective, but hypoxanthin and theobromine were inactive.

Approaching the problem from a different angle, Moore and Wang⁸ considered that the substance responsible for the brown pigmentation of the uterus of the vitamin E-deficient rat resembled a "melanin" formed by the

* The author wishes to acknowledge the valuable criticism of Dr. L. J. Harris and the technical assistance of Mrs. Aileen Bright, Mr. B. J. Milton, and Miss Pamela Holder.

acid hydrolysis of proteins in its fluorescence and solubility products. Later, the possibility of changes involving the oxidation and deamination of tryptophane was entertained, and it was suggested that vitamin E, among its other functions, may prevent the abnormal oxidation of protein.⁹

The present experiments were carried out in order to confirm and extend previous observations on the effects of combined deficiency of vitamin E and protein on rats. Conditions were so chosen that both deficiencies occurred in severe form simultaneously.

Experimental

Experiment 1. The Interaction of Vitamin E and Protein during Early Growth

Preliminary Period. Young female albino rats weighing 40–46 g. were given a diet deficient in vitamin E, which at first contained casein (vitamin free, Glaxo) 25 per cent, cane sugar 50 per cent, lard 10 per cent, dried yeast 10 per cent, and minerals 5 per cent. Vitamins A and D were supplied as one drop of halibut-liver oil, and vitamin K as 50 μ g. of 2 methyl 1,4,naphthoquinone weekly. As the rats reached predetermined body weights of 60, 70, 80, and 90 g., their casein intakes were progressively reduced to 12, 6, 3, and 0 per cent of the diet, with the substitution of corresponding amounts of sugar. These reductions were so timed that the rats stopped growing after about 6 weeks from the beginning of the experiment as they approached 100 g. in weight, with the dried yeast component of their diet as the only source of protein. At this point, depigmentation of the incisor teeth was just beginning to be noticeable, although the teeth of control animals given α -tocopherol remained normal.

Experimental Period. After about 10 weeks from the beginning of the experiment, 4 groups of 5 animals each were selected. Rats in Group 1 were each given, during the morning, a mixture of casein 2 g., sucrose 0.5 g., yeast 1 g., lard 1 g., and minerals 0.5 g., and later in the day were allowed sucrose *ad lib*. Daily doses of 1 mg. of dl- α -tocopherol were also administered, half being given as the free alcohol and half as the acetate. Group 2 were fed similarly, but without tocopherol. Group 3 received only 0.2 g. of casein, mixed with sucrose 2.3 g., yeast 1 g., lard 1 g., and minerals 0.5 g., supplemented by additional sucrose and tocopherol. Group 4 received the same treatment without tocopherol. All groups continued to receive adequate doses of vitamins A, D, and K.

Growth. From FIGURE 1 it will be seen that during the next 13 weeks equally good growth occurred in Groups 1 and 2, both of which received liberal amounts of casein. Growth was poor in the groups given inadequate allowances of protein, but was slightly better in Group 3, which received tocopherol, than in Group 4.

Teeth. FIGURE 2 shows the incisor teeth of 4 rats out of each group as they appeared at the end of the experiment. The normal brown pigmentation was completely restored in Groups 1 and 3, which both received tocopherol. In Group 4, deficient both in tocopherol and casein, all the teeth

HIGH CASEIN

LOW CASEIN



FIGURE 1. The interrelation of vitamin E and protein in promoting growth in young rats.

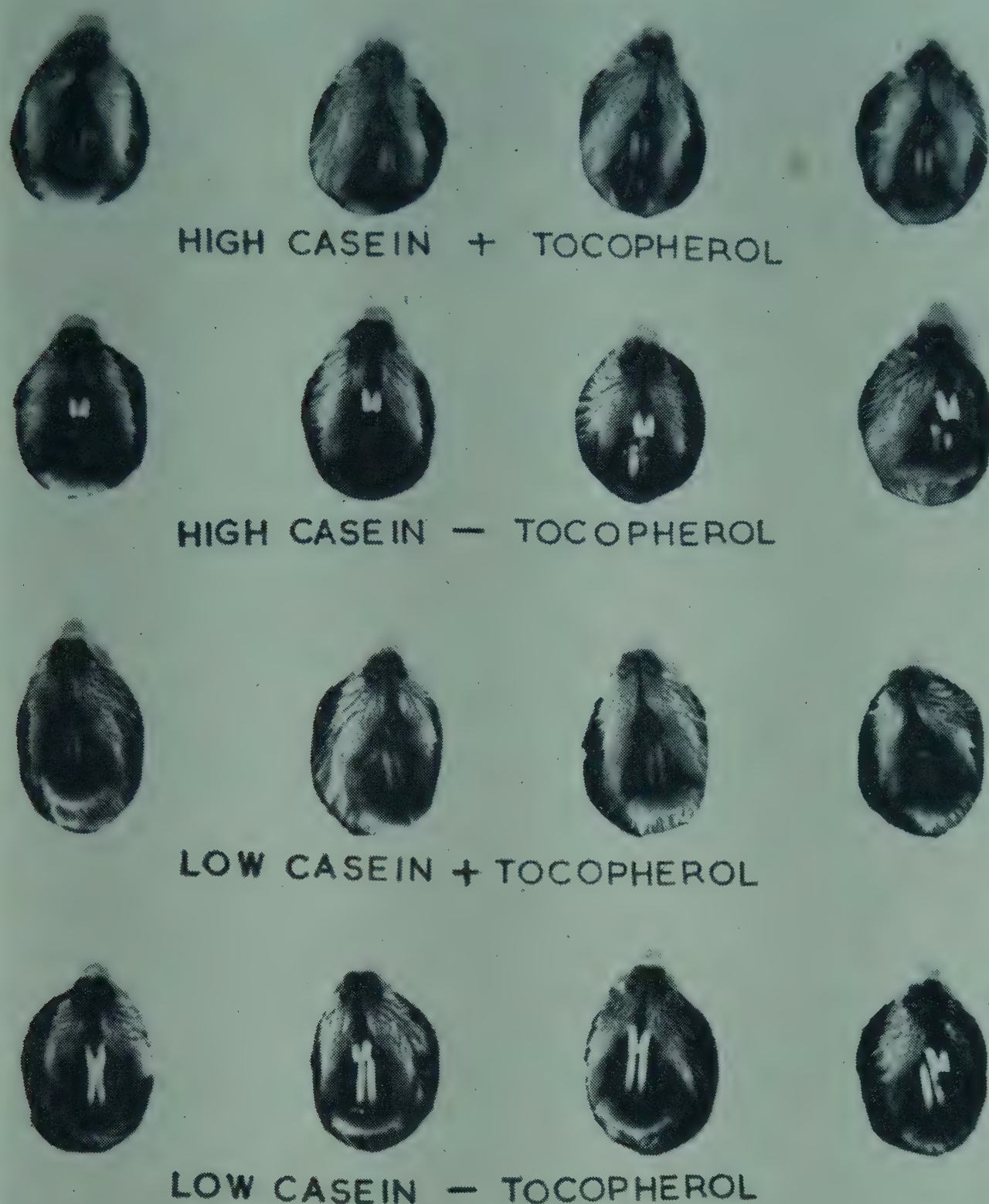


FIGURE 2. The interrelation of vitamin E and protein in preserving dental pigmentation.

were completely white. In Group 3, adequate in protein but deficient in vitamin E, the upper teeth were white, while the lower teeth were mainly brown, although slightly mottled with white in two of the animals.

Uteri. In 5 spare rats which were examined after receiving the diet deficient in vitamin E, and eventually with no casein, for periods of 9–16 weeks, the uteri were under-developed, but were virtually normal in color and fluorescence. In previous work,⁹ the uteri of rats kept for 10 weeks on a diet deficient in vitamin E, but adequate in protein, showed a decided yellow-brown fluorescence under ultra-violet irradiation, although only slight discoloration could be noticed in visible light. The deficiency in protein appears, therefore, to have arrested the tendency to uterine pigmentation.

At the conclusion of the main experiment, the uteri of all the rats were

examined after they had received the experimental diets for total periods of 22–25 weeks. In Groups 2 and 4, which were not dosed with tocopherol, the uteri appeared only slightly brown in visible light, but showed a marked yellow-brown fluorescence under irradiation. In Groups 1 and 3, which were dosed with tocopherol, the uteri were normal in color and fluorescence.

Experiment 2. The Interaction of Vitamin E and Protein in Adult Rats

Preliminary Period. Young female albino rats weighing 33–82 g. were kept on a diet deficient in vitamin E and containing 25 per cent of casein, as used in the preliminary stages of the preceding experiment. The progress of deficiency was studied by inspection of the teeth. At the beginning of the experiment, the normal brown pigmentation had not developed in some of the more immature animals, but appeared during the next 2–4 weeks. Subsequently, depigmentation of the upper incisors occurred in all animals at times 5–10 weeks after the beginning of the experiment. In a rat killed after 10 weeks of restriction, the uterus appeared a faint brown color in visible light and had an orange-brown fluorescence under irradiation. After the animals had received the deficient diet for 26 weeks, body weights of 176–234 g. had been attained and, in most instances, had changed little during the preceding 10 weeks. The upper incisor teeth all became completely white, except for a faint brown mottling in one tooth in each of two animals. The lower teeth were usually brown, but in some instances were mottled brown and white.

Experimental Period. The animals were next divided into 4 groups of 4 rats each. Group 1 continued to have the same basal diet, but supplemented with daily doses of tocopherol. Group 2 received the basal diet only. Group 3 were given a basal diet in which all the casein was replaced by additional sucrose, together with supplements of tocopherol. Group 4 received the diet without either casein or tocopherol. Food was given to all groups *ad lib.* Except for rats which died or were killed for examination, the experimental period lasted for 32 weeks.

Weight Changes. The weight changes are shown in FIGURE 3. In Group 2, which continued to receive the same diet as during the preliminary period, the body weights remained virtually unchanged or increased slightly. In Group 1, which received both casein and tocopherol, considerable increases in weight were observed. In Group 4, deficient in both casein and tocopherol, the animals fell steadily in weight. All of them eventually died, or were killed in a moribund condition, within 20–30 weeks of the removal of casein from their diet. In Group 3, deficient in casein but supplemented with tocopherol, the body weights for about the first 15 weeks of the experimental period fell at about the same rate as in Group 4. Later, however, the decline in body weight was arrested. In one rat, the weight remained almost stationary for many weeks, while the 3 other animals began to gain in weight.

Teeth. In Groups 1 and 3, which received tocopherol, the reappearance

VITAMIN E AND PROTEIN IN ADULT RATS.

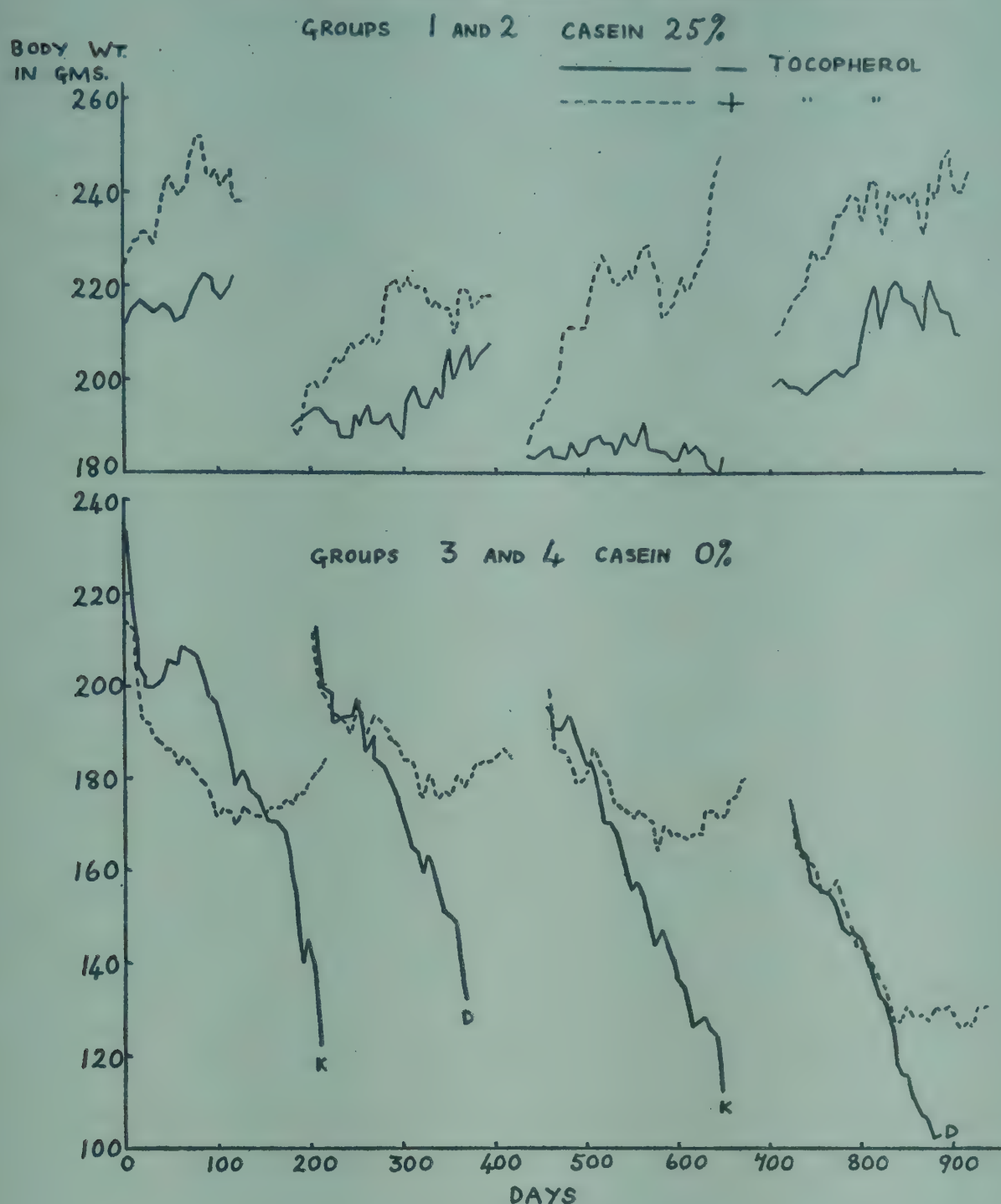


FIGURE 3.

of dental pigmentation could be noted after dosing for 4-7 weeks. The teeth eventually resumed their normal brown color in every animal. In Group 4, receiving neither tocopherol nor casein, the lower teeth followed the upper teeth in becoming completely white, with the exception of one slightly brown tooth in one animal. In Group 2, which were given casein but not tocopherol, the upper teeth remained white in 3 animals, while, in the remaining animal, one tooth was brown and one mottled. The lower teeth in this group were brown in 3 animals and mottled brown upon white in the remaining animal.

Uteri and Skeletal Musculature. As the result of the prolonged preliminary period on the diet deficient in vitamin E, the uteri were brown even in those groups which later received tocopherol. The uteri in the Group 2, which received casein but no tocopherol, were somewhat darker in color and larger in size than in the other groups.

In Groups 1, 2, and 3, the skeletal muscles were normal in appearance.

In some of the animals of Group 4, however, which were deficient in both vitamin E and protein, the muscles appeared light brown in visible light and fluoresced yellow-brown under irradiation. A similar picture had been seen by Moore and Wang¹⁰ in rats kept for very long periods on a diet deficient in vitamin E but adequate in protein. In view of the hepatic injuries observed (as reviewed here subsequently), the possibility of complications through the staining of the tissues with bile pigments should not be overlooked.

Anaemia. During life, all the rats in Group 4, without casein or tocopherol, appeared to be anaemic, and this impression was confirmed by the appearance at autopsy. Since this abnormality had not been expected, no blood counts were made on the first three animals to succumb. The remaining rat, however, was found to have 3.4 million R.B.C. and 67,000 W.B.C. per mml., with haemoglobin within the normal range. In contrast, the animals of Groups 1, 2, and 3, which did not appear anaemic while alive, had 5.6–6.9 R.B.C. and 10,900–32,700 W.B.C.

Liver Lesions. At autopsy, the livers in Groups 1 and 2, receiving casein, with or without tocopherol, appeared normal to naked-eye inspection in size, color, and texture. The livers in Group 4, deficient in both protein and tocopherol, were abnormal in appearance and, in two instances, appeared much lighter in color in some parts than in others. When they were examined (very kindly) by Professor H. P. Himsworth, massive necrosis, either of early or recent origin, was detected in three instances, with fatty infiltration and evidence of early portal fibrosis. Special staining indicated the presence of iron in the parenchyma and macrophages. In Group 3, deficient in protein but dosed with tocopherol, the livers were all enlarged, making up an average 7.2 per cent of the body weight, as compared to 4.4–5.9 per cent in the other groups. They were a uniform flesh color, instead of dark red as in the groups receiving casein, and were very fragile when handled. Fatty infiltration was confirmed by chemical analysis by Mr. I. M. Sharman, the fat contents being 12.8–33.0, mean 24.8 per cent. The liver of the only rat which had failed to regain weight during the final stages of the experiments contained the lowest level of fat and was less abnormal than the livers of the other animals in superficial appearance. In spite of the absence of anaemia, iron was detected in the livers of these animals.

Experiment 3. Attempts to Produce Brown Fluorescent Pigment from Protein Derivatives

In vitro. Moore and Wang^{8, 9} found that brown fluorescent pigments, somewhat resembling the material which they had obtained from the tissues of vitamin E-deficient rats, could be prepared by the oxidation of protein in acid solution. With tryptophane, but not with other amino acids which were tested, intensely brown derivatives with yellow fluorescence were readily obtained by dissolving in glacial acetic acid and heating with a drop of concentrated sulphuric acid followed by an oxidising agent, such as

benzoyl peroxide. The pigment so obtained, however, differed from that separated from the tissues of rats in not being extractable from an acid aqueous medium with isopropyl alcohol. β -Indolyl acetic acid gave similar pigmented derivatives after treatment with dilute sulphuric acid, and, in the absence of the amino group, the products were extractable with isopropyl alcohol. Skatole gave pigmented products on oxidation, even in the absence of acid, and became slightly brown colored on treatment in chloroform solution with rancid fat. The pigment had the same solubility properties as the natural pigment.

In vivo. Attempts to cause pigmentation in rats deficient in vitamin E by giving large doses of tryptophane, or of β -indolyl acetic acid, were unsuccessful. When skatole was incorporated in the basal diet, daily doses of up to about 50 mg. had no apparent ill effects, but daily doses of 100 mg. were toxic. At autopsy, no abnormal pigmentation was observed.

When skatole in solution in lard was injected subcutaneously, fat, with the bluish fluorescence of skatole, could be recovered from the sites of injection at autopsy some weeks later. The surrounding tissues were faintly brown in parts and fluoresced a weak yellow-brown under irradiation. Injections of plain lard, however, caused similar discoloration and fluorescence.

Piebald animals were used for these experiments, and they were not given the diet deficient in vitamin E until about 70 g. in weight. After 15 weeks of restriction, the uteri were brown, but the incisor teeth still retained their normal pigmentation.

Discussion

The results of the above experiments support the view that nutritional requirements for protein and for vitamin E are to some degree interrelated. Certain minor points of difference from the findings of previous workers, however, are perhaps worth mentioning. Thus, Dam¹ found that tocopherol merely prolonged the survival of rats deficient in protein, while in the present work it allowed animals equally deficient in protein to survive indefinitely. Hove and Harris⁵ found that the efficiency of utilisation of protein was only increased by tocopherol when it was present in the diet within the limits of 6–12 per cent. In one of the present experiments, tocopherol improved the growth of rats receiving a diet with 25 per cent of protein. These divergencies were due, presumably, to the use of rats differing in age and size.

The term "interrelation," as has already been pointed out,¹⁰ covers a wide range of meaning. The nutrients concerned may participate in the same physiological or biochemical systems or may be associated in the development or maintenance of the same tissues; or, one may merely influence numerically the body's requirement of the other. It might perhaps be argued that, since both vitamin E deficiency and protein deficiency are injurious to rats, the condition of an animal suffering from the combined deficiencies must generally be worse than when a single deficiency is suffered. Treatment of the doubly deficient animals with either of the missing factors

should, therefore, cause some improvement, but this will not prove that the two nutrients are related in physiological or biochemical systems. Thus, a careful review of the evidence from all aspects is necessary before deciding what significance can be attached to the apparent interaction between tocopherol and protein.

Growth. If vitamin E spares protein by increasing the efficiency of its utilisation, this effect should be plainly shown when the intake of protein is low but less apparent when a liberal intake allows "waste" of protein, without this nutrient becoming a limiting factor for growth. The results of Experiment 1, on rats in the early stages of growth, support this conclusion, but in Experiment 2, on adult rats, vitamin E caused weight increases even when the protein intake was adequate.

Although vitamin E affected growth, its influence was obviously secondary to that of protein, at least in the early stages of development. Thus, rats provided with adequate casein grew rapidly, in spite of the plain evidence of vitamin E deficiency to be seen in their dental abnormalities. If we conclude that there is a "time lag" of some weeks between the commencement of the biochemical lesions due to vitamin E deficiency and the outward manifestation of their effects on the teeth, as might be inferred from the corresponding interval observed between the commencement of dosing with vitamin E and the return of the teeth to normal (as will be shown), the relative effects of protein and vitamin E upon growth are seen in striking contrast. Although the biochemical effects of avitaminosis E must commence soon after the restriction of the animals to the deficient diet, and probably within the first few days, vigorous growth continues for many weeks if an adequate intake of protein is allowed.

Teeth. In the present experiments, rats dosed with tocopherol always had normally pigmented incisor teeth. The only exceptions were seen in two spare rats in Experiment 1, which were dosed with tocopherol prophylactically, and which had white teeth for a short period some weeks after casein was first omitted from their diet. Evidence of interaction of protein and vitamin E was seen in the condition of the teeth of the animals deficient in tocopherol but adequate in casein. In both experiments the upper teeth were usually white, while the lower teeth were brown. In contrast, rats deficient in both vitamin E and protein had all their teeth completely white. According to the evidence of the lower teeth, therefore, an interaction between the two nutrients may be inferred, although the evidence of the upper teeth would not justify this conclusion!

In Experiment 2, in which the rats were deprived of vitamin E for a prolonged period before different levels of protein were allowed, periods of 5-7 weeks usually elapsed before depigmentation could be detected in the erupted areas of the upper teeth. Restoration of pigmentation occurred at about the same interval after dosing with tocopherol. In rats which were given neither tocopherol nor casein, depigmentation of the lower teeth only became apparent after even longer intervals.

Uteri. The evidence of Experiment 1 suggests that when the development of the uterus is prevented by deficiency of protein the formation of brown pigment is also prevented. Therefore, an adequate protein allowance seems necessary for the appearance of this abnormality, but it would seem unjustifiable to interpret this finding as necessarily implying a close inter-relationship between protein and vitamin E.

Other Lesions. Perhaps the strongest evidence of the interaction is to be found in the ability of either casein or tocopherol to allow rats to survive for prolonged periods when they are grossly deficient in the other of these two nutrients. The ability of tocopherol to prevent liver damage due to protein deficiency has been demonstrated in experiments under various conditions by several previous workers.^{6, 11, 12} In the present work, tocopherol did not protect against hepatic abnormality in protein deficiency, since the animals in Experiment 2, Group 3, all showed severe fatty infiltration of the liver. It is possible that the vitamin prevents the superimposition of necrosis upon fatty infiltration, as occurred in some of the animals in Group 4. Either tocopherol or casein protected the animals against the severe anaemia which occurred, according to blood counts on one animal and the superficial examination of others, when the diet was deficient in both nutrients.

The Relative Rate of Development of Abnormalities in Vitamin E Deficiency. In the experiments in which the growth of young rats deficient in vitamin E was checked by a simultaneous deficiency of protein, the incisor teeth became white although the uteri remained normal in color. In the rats used for the attempts to produce brown pigmentation by the injection of skatole, and in a corresponding control animal, the teeth remained normal although the uteri became deeply pigmented. It is plain, therefore, that these lesions proceed independently in their development and that the degree of emphasis on each abnormality will depend on the exact experimental conditions.

A noteworthy feature in all the experiments described was the freedom of the animals from paralysis. Since they had been bred from mothers receiving diets adequate in vitamin E, the rapid appearance of this affliction was not to be expected. Early signs of in-coordination, however, might perhaps have been expected in some of the animals which received the deficient diet for over a year. At one time, paralysis was frequently observed in rats in this laboratory kept for long periods on diets deficient in vitamin E.¹³

Summary

(1) Evidence of the interrelation of vitamin E and casein in the nutrition of rats was obtained in experiments in which diets either adequate or inadequate in protein were given, in each case with or without supplements of tocopherol.

(2) Protein had a predominating influence on the early stages of growth, while vitamin E had a predominating influence of the maintenance of normal dental pigmentation. Vitamin E, however, improved early growth

when the protein intake was moderately low. An adequate allowance of casein partially corrected dental depigmentation in the absence of vitamin E.

(3) When the allowance of protein was very low, rats not dosed with tocopherol declined continuously in weight, became anaemic, and eventually succumbed with necrotic liver lesions. The same initial rate of decline was shown by rats dosed with tocopherol, but, later, the fall in weight was checked and, in most cases, there was some recovery towards the initial weight. Although the dosed animals remained in superficially good health, all were found to have severe fatty infiltration of the liver when killed at the end of the experiment.

(4) Brown fluorescent products, somewhat resembling the pigment found in the tissues of rats deficient in vitamin E, could be obtained from tryptophane, β -indolyl acetic acid, or skatole by chemical treatment. Deficient rats, however, showed no tendency to increased pigmentation when they were given large amounts of these substances.

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COMPARISON OF A FATAL TOCOPHEROL DEFICIENCY DISEASE IN RATS WITH THE SYNDROME CAUSED BY CCl_4

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Rats fed vitamin E-free diets low in casein† display several abnormalities which can be prevented either by alpha-tocopherol or by raising the casein level of the diet. Criteria used to demonstrate this interrelation between vitamin E and casein have been:

- (a) Body weight loss in adult rats fed a 5 per cent casein diet, low in vitamin E, for six months.¹
- (b) Blanching of the incisor teeth of rats.¹
- (c) Efficiency of food utilization for growth of weanling rats on a 10 per cent casein diet low in vitamin E.²
- (d) Resistance of rats to acute CCl_4 toxicity when they are fed a 10 per cent casein diet low in vitamin E.³
- (e) Sudden death with massive lung hemorrhage and liver necrosis in rats on a 10 per cent casein diet low in vitamin E.⁴

With the demonstration of the vitamin E-casein interrelation, two questions came to mind. First, has a "true" vitamin E deficiency been produced in rats, or does the tocopherol "spare" some essential nutrient furnished in minimal amounts by the low casein level? Second, what is the nature of the dietary component of casein with which vitamin E interrelates? It can be hoped that answers to these questions will greatly advance knowledge on the physiological function of vitamin E and, possibly, help to explain such species differences as, for example, between the rabbit that dies within a month on a diet complete in all nutrients except vitamin E, and the rat that will thrive for over a year on the same diet.

Before proceeding with the report on the relation of the CCl_4 toxicity to a vitamin E deficiency, it would be well to review the rôle of sulfur amino acids, and other possible limiting factors in casein, as nutrients with which alpha-tocopherol may interrelate physiologically. It has been reported¹ that the addition of 1 per cent of tryptophan, valine, arginine, or lysine to the 5 per cent casein diet did not benefit rats in the absence of alpha-tocopherol; but a slight response was seen with l-cystine added to the diet. This experiment has been repeated in a strictly identical manner, with and without l-cystine added at a 0.5 per cent level (Hove, unpublished data from Distillation Products, Inc.). The results of this study are given in TABLE 1. The beneficial effect of alpha-tocopherol in minimizing loss in body weight was as great or greater on the cystine-supplemented diet as on the basal diet, even though cystine by itself was quite effective. The absence

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† The diets used by the author have contained the stated casein level, salt mixture 4, lard 19, U.S.P. cod-liver oil 1, and sucrose to 100. Pure vitamins were added to supply the following levels per gram of diet: thiamine, riboflavin, pyridoxine, 5 μg . each; calcium pantothenate 20 μg ., niacin 40 μg ., choline chloride 2 mg., i-inositol 0.2 mg., and menadione 2 μg .

TABLE 1

THE INFLUENCE OF α -TOCOPHEROL AND L-CYSTINE ON ADULT RATS* RESTRICTED FOR 26 WEEKS TO 8.9 GRAMS OF DIET DAILY

Diet	Daily α -tocopherol (mg.)	Body weight loss (% \pm S.E.)	Testes (% of final body wt. \pm S.E.)	Liver (% of final body wt. \pm S.E.)
Casein, 5%	0	33.5 \pm 3.33	0.68 \pm .046	4.27 \pm .09
	1	20.2 \pm 1.50	1.07 \pm .049	3.40 \pm .32
Same plus 0.5% l-cystine	0	19.1 \pm 2.76	0.71 \pm .045	7.06 \pm .59
	1	7.6 \pm 0.97	0.88 \pm .019	5.70 \pm .04

* Six male rats per group with initial weights varying from 250 to 266 grams.
† *t* for P = .01 is 3.17, and for P = .05 is 2.23 for ten degrees of freedom. Therefore, all differences due to alpha-tocopherol are significant statistically.

of a relation between vitamin E and cystine is emphasized further by the data on testes and liver size. Atrophy of the testes was not prevented by cystine but was prevented by alpha-tocopherol. Hypertrophy of the liver was evident in the rats not receiving vitamin E whether cystine was present or not. The fat content of these livers (expressed as per cent of fresh tissue) was 4.7 per cent without tocopherol, 5.3 per cent with tocopherol for rats on the basal diet, and 5.8 and 6.9 per cent, respectively, for the rats on the diet plus cystine. No evidence of liver damage was seen by gross inspection.

Blanching of the incisor teeth was complete in all of the vitamin E-deficient rats of TABLE 1, whether receiving cystine or not, while a completely normal tooth color was present in all of the tocopherol-supplemented animals.

When measuring the efficiency of food utilization by weanling rats on a 10 per cent casein diet, supplements of alpha-tocopherol were found² to be beneficial whether or not l-cystine was added to the diet at a 0.5 per cent level, although the cystine itself produced a considerable increase in efficiency.

The data obtained with the first three criteria, as listed previously, tend to deny any vital relationship between cystine and alpha-tocopherol metabolism. Using the acute CCl₄ toxicity as a criterion, it was found³ that dl-methionine added at a 0.1 per cent level to the 10 per cent casein diet could completely replace vitamin E. Theophylline shared this property; xanthine, histidine, gamma-tocopherol and guanine were but moderately effective. Unfortunately, cystine has not yet been tested in this type of experiment.

Considering next the syndrome of sudden death with gross damage to the lungs and liver, it is evident from the discussion to follow that the precise rôle of sulfur amino acids is unclear. Himsworth and co-workers⁶ first described massive hepatic necrosis in rats as the result of a cystine (or methionine) deficiency. Schwarz⁵ was unable to demonstrate an effect of either cystine or methionine in preventing massive hepatic necrosis, although

alpha-tocopherol was protective (but not gamma-tocopherol as supplied in soybean oil). György and Goldblatt⁷ found both cystine and methionine to be somewhat protective in that the incidence of hepatic necrosis was lessened but not prevented entirely. Hove, Copeland, and Salmon⁴ noted that l-cystine added at a 0.1 per cent level to the 10 per cent casein diet low in vitamin E reduced the incidence of the fatal syndrome from 75 to 40 per cent and delayed the time of onset. However, dl-methionine, at the same level, was without benefit and was even ineffective when added at a 1 per cent level to a 16 per cent oxidized casein diet. Theophylline, xanthine, and histidine were ineffective in preventing the acute syndrome of lung hemorrhage and liver necrosis. The fatal syndrome was produced in rats fed vitamin E-low diets containing 10 per cent casein and varying in fat from 5 to 40 per cent.

In a convincing paper, Dam and co-workers⁸ reported studies on the relation between l-cystine and alpha-tocopherol in the nutrition of the chicken. They found that on a low-protein, high-fat diet exudative diathesis in chicks could be prevented by vitamin E, or by cystine, vitamin C, or nordihydroguaiaretic acid. However, chicks on the encephalomalacia-producing, high casein diet were consistently protected only by alpha-tocopherol. The beneficial effect of cystine against exudate production was ascribed to its protein-sparing action.

In attempting a summary of the data on the relation of cystine (or methionine) to the metabolism of vitamin E, it may be stated, tentatively, that an interrelation exists in those conditions which depend upon the integrity of the walls of vascular vessels. Included in this class are the exudative diathesis in chicks and the fatal lung hemorrhage-liver necrosis syndrome and, possibly, the CCl₄ toxicity in rats. The frequently contradictory data may simply illustrate the sensitivity of such a criterion to slight changes in environmental and dietary conditions. Because of these contradictory results and because cystine appeared to be unrelated to vitamin E in any simple sense when considering criteria such as blanching of teeth, body weight, and food utilization, it must be concluded that a deficiency of sulfur amino acids is not the whole answer to the question of why low dietary casein increases the need of the rat for vitamin E.

Any of the criteria just listed might be employed in the attempt to establish the nature of the nutrients present in casein with which alpha-tocopherol interrelates. However, the method involving resistance to the lethal action of CCl₄ gives results so quickly and with such sensitivity that this would seem to be the criterion of choice, other factors being equal. With this in mind, a program has been evolved which contemplates an attempt to establish whether the CCl₄ toxicity does, in fact, induce a true vitamin E deficiency in rats that have been conditioned on a low dietary casein regimen. Two phases of this program have been carried out and will be reported here. The first phase deals with a comparison of the lesions seen in chronic CCl₄ toxicity with those evident in rats that died suddenly of a lung hemorrhage-liver necrosis syndrome resulting from maintenance on a vitamin E-low diet.⁴ The second phase concerns the influence of CCl₄ on the creatine-

creatinine excretion pattern and the effect of alpha-tocopherol on this excretion.

Weanling rats were placed on the vitamin E-free diet containing 10 per cent casein. Two-thirds of the animals received 0.05 cc. of CCl₄ in olive oil per week by stomach tube. This level of CCl₄ is about one-fourth of the mld. Nearly half of the rats treated with CCl₄ received, in addition, 7 mg. of dl-alpha-tocopherol per week. The results are given in TABLE 2.

TABLE 2
THE INFLUENCE OF CCl₄ FED AT .05 CC. PER WEEK PER RAT, ON THE FATAL VITAMIN E DEFICIENCY AMONG WEANLING RATS RECEIVING THE 10 PER CENT CASEIN BASAL DIET

	Supplements of α -tocopherol	
	none	7 mg./wk.
Number of rats	18	15
Number of spontaneous deaths	15	0
Average number of days on diet	31	45
Liver (% of body weight)	4.9	4.7
Testes (% of body weight)	1.5	1.9
Number of rats with gross liver damage	12	0
Number of rats with massive lung hemorrhage	9	0
Growth rate (gm./rat/day)	0.8	1.4

All but 3 of the vitamin E-deficient rats getting CCl₄ died after an average time of 31 days on experiment. The 3 survivors were killed for examination. None of the tocopherol-supplemented rats died, but they were killed for examination 2 weeks after each of their litter mates had died. At necropsy, the vitamin E-low animals treated with CCl₄ showed a high incidence of massive lung hemorrhage and liver necrosis. These lesions were identical, both grossly and histologically, with the lesions seen in the acute vitamin E deficiency disease.⁴ The only difference was in the time of onset of the fatal condition. The CCl₄-treated rats survived 31 days, on the average. But without CCl₄ the same condition developed only after an average time of 70 days (Hove, Copeland, and Salmon⁴). The teeth of the rats given CCl₄ showed signs of blanching considerably sooner than those of the vitamin E-deficient rats not receiving CCl₄.

The conclusion drawn from these data is that CCl₄ precipitates a fatal disease in rats that is identical in all observed respects to that produced by a vitamin E deficiency among rats on a 10 per cent casein diet. Supplements of alpha-tocopherol prevent this fatal syndrome from occurring among rats receiving either treatment. From this it may be postulated that the mechanism of action of CCl₄ is to induce a vitamin E deficiency.

To test further the hypothesis that CCl₄ induces a vitamin E deficiency, attention was turned to the creatine and creatinine-excretion pattern. One of the well-established symptoms of a vitamin E deficiency, in animals on an otherwise normal diet, is a high urinary excretion of creatine. In the rat,

the creatinurea develops slowly through several months, even in the absolute absence of vitamin E. Therefore, this animal is an excellent subject for the study of creatine excretion as influenced by CCl_4 , either with or without supplements of alpha-tocopherol.

The influence of CCl_4 on the creatine and creatinine-excretion pattern was determined on weanling rats fed the 10 per cent casein diet. After 5 weeks on this diet, 16 male rats were divided into 4 groups (individually caged) and given alpha-tocopherol supplements on the following schedule: 0, 0.5, 2.5, and 10 mg. daily for a 10-day period. Two days after the end of the 10-day supplementation period, a single dose of CCl_4 was injected intraperitoneally at a level of 1 cc. per kg. body weight. The 24-hour creatine and creatinine excretion was determined before the injections and at intervals during the following week.

The changes in the average creatine excretion are shown graphically in FIGURE 1. It is apparent that the CCl_4 injection produced a profound

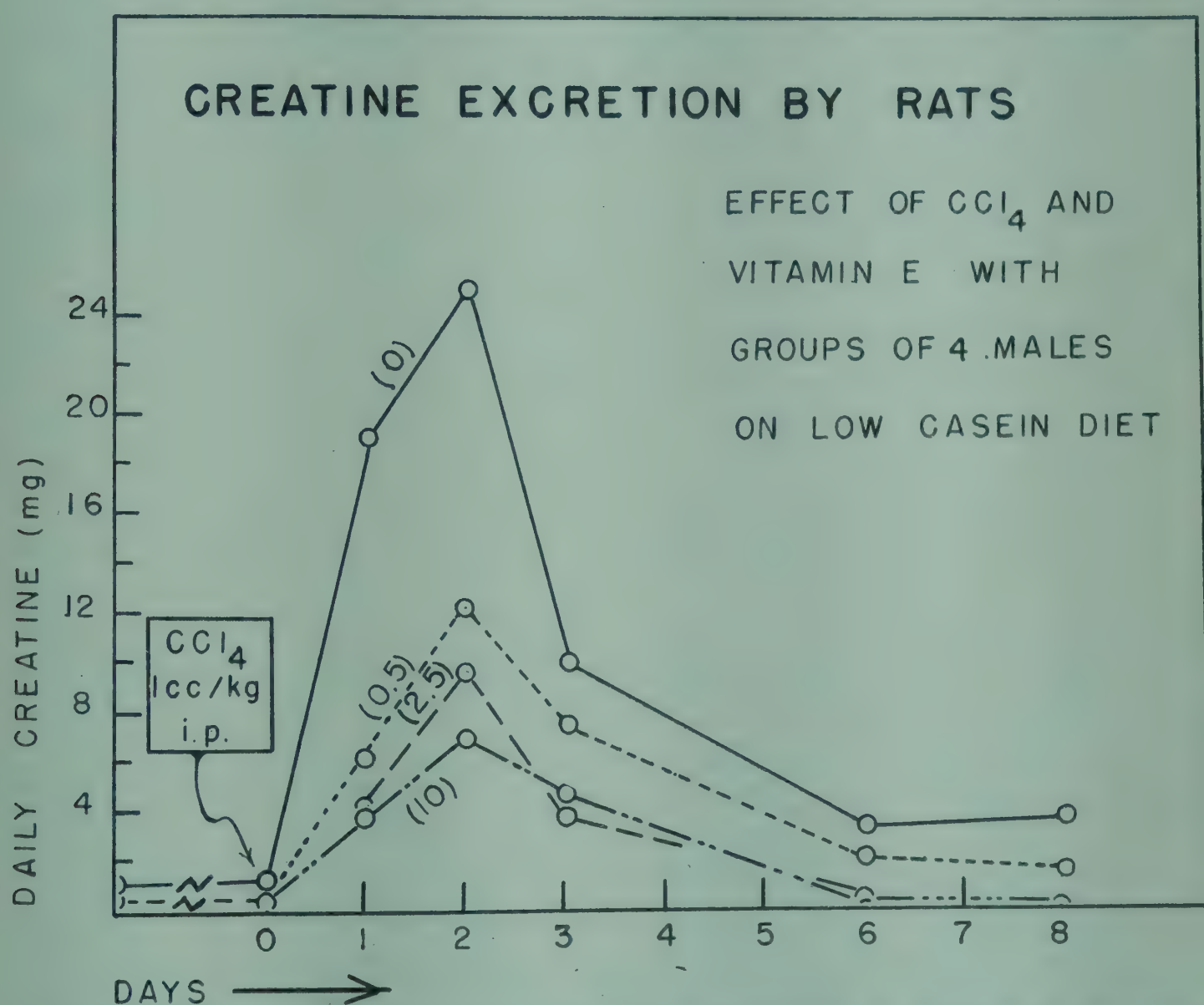


FIGURE 1. Creatinurea in rats caused by the intraperitoneal injection of 1 cc. of CCl_4 per kg. body weight and the protective effect of alpha-tocopherol supplements given at levels of 0.5, 2.5, and 10 mg. daily for ten days prior to injection of CCl_4 . Each value is the average of four male rats after six weeks on the basal diet.

creatinurea that rose to a peak by the second day and then subsided. In the animals which had received tocopherol supplements there was a progressive lowering of the creatinurea peak, and the creatine excretion dropped to normal rapidly after the peak was passed. The creatine excretion of the vitamin E-free rats never returned to its original low level.

The average creatinine excretion of the rats from the same experiment is shown in FIGURE 2. From these data it is evident that CCl_4 produced a

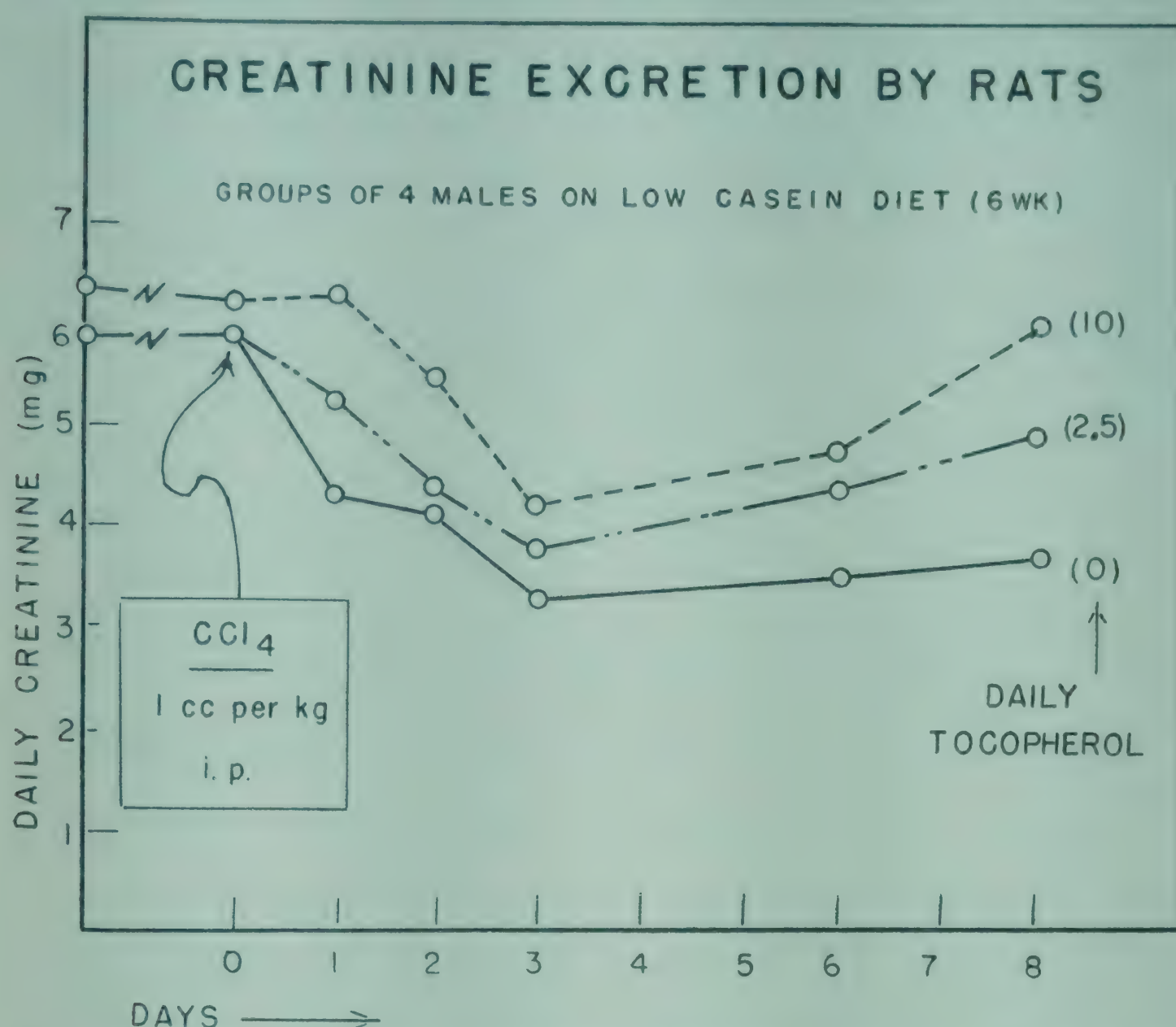


FIGURE 2. Hypo-creatininurea in rats caused by the intraperitoneal injection of 1 cc. of CCl_4 per kg. body weight, and the protective effect of alpha-tocopherol supplements given for 10 days prior to injection of CCl_4 .

sharp drop in the creatinine excretion down to a minimum value on the third day. In rats which had received tocopherol the creatinine fall was not as pronounced, and after the third day a gradual recovery toward the normal value took place.

For purposes of comparison, the pattern of nitrogen excretion in chronic vitamin E-deficient rats was determined by standard methods on 24-hour urine samples. The rats had been kept for 8 months on a vitamin E-free diet containing 18 per cent casein. The data in TABLE 3 were obtained on 12 vitamin E-deficient rats and 12 control rats receiving 7 mg. of dl-alpha-tocopherol weekly. Similar data could not be obtained from rats receiving the 10 per cent casein diet because, as previously reported,⁴ such rats usually died with massive lung hemorrhage and hepatic necrosis after 2 to 3 months. It is evident from TABLE 3 that chronic vitamin E deficiency in rats is associated not only with a creatinurea but also with a decreased creatinine excretion. The urinary excretion of total nitrogen and alpha-amino nitrogen was not influenced when calculated on the basis of food consumption.

In summarizing this phase, it has been demonstrated that single sublethal

TABLE 3

URINARY EXCRETION OF NITROGENOUS COMPOUNDS BY CHRONIC VITAMIN E-DEFICIENT RATS WHICH HAD BEEN RESTRICTED TO AN 18 PER CENT CASEIN DIET FOR 8 MONTHS

	<i>No tocopherol</i>	<i>Plus tocopherol</i>
Number of rats	12	12
Average body weight, g.	401	468
Average daily food intake, g.	13.1	15.4
	(mg./24 hrs.)	(mg./24 hrs.)
Creatine	7.0	0.9
Creatinine	8.5 \pm .93*	13.4 \pm 1.07*
Alpha-amino nitrogen	1.09	1.23
Total nitrogen	190	221

* Standard error of the mean.

doses of CCl_4 resulted in a creatinurea and in a fall in the creatinine excretion of rats. Both of these conditions attained greatest severity by the second or third day, and in both cases the rats which had received alpha-tocopherol showed a diminished severity and a more rapid return to normal values. These facts may be closely related to earlier observations³ that nearly all deaths following lethal doses of CCl_4 occurred during the second day and that supplements of alpha-tocopherol were dramatically effective in protecting against death due to CCl_4 . The effect of tocopherol was evident only when the rats had received a 10 per cent casein diet. On a diet with a normal casein level, vitamin E was without benefit. Possibly, CCl_4 interferes with a biochemical reaction for which both alpha-tocopherol and some dietary component present in casein are needed.

The creatinurea and hypo-creatininurea due to CCl_4 were considerably more pronounced than observed in the chronic vitamin E-deficient rats, but this may simply represent the difference between a chronic and an acute stage. Practically nothing is known about the mechanism of CCl_4 toxicity, the physiological function of vitamin E, or the significance of deranged creatine metabolism in these two conditions. If an altered creatine metabolism, correctable by alpha-tocopherol, is accepted as a valid criterion for the vitamin E-deficient state, then the data presented here are consistent with the hypothesis that at least one of the toxic manifestations of CCl_4 is in inducing an acute vitamin E deficiency in rats.

Summary

An attempt has been made to establish whether a CCl_4 toxicity induces a true vitamin E deficiency in rats fed a 10 per cent casein diet.

It was found that young rats given .05 cc. of CCl_4 per week died after an average of 31 days on experiment. Similar rats on the same diet, but not receiving CCl_4 , died suddenly at an average time of 71 days on diet. In both cases, death was preventable by supplements of alpha-tocopherol, and the lesions seen at necropsy were identical grossly and histologically. Massive lung hemorrhage, liver necrosis, blanching of the incisors, and lower growth rate were noted.

In a second set of experiments, it was found that single, sublethal injections of CCl_4 into rats produced a creatinurea and a hypo-creatininurea. Chronic vitamin E deficiency produced a similar, but less marked, excretion pattern. In both cases, supplements of alpha-tocopherol substantially corrected this abnormal excretion.*

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Discussion of the Paper

DOCTOR Z. MENSCHIK: Doctor Hove mentioned that his rats which received tocopherol for several weeks showed an increase of fat in the liver. I would like to add that, during experiments performed by my co-workers and myself on mice, we noted a considerable increase in the neutral fat content of the livers of animals receiving vitamin E. The livers, in these instances, showed a peripheral distribution of fat similar to the fatty changes described by Noël (*Recherches histophysiologiques sur la cellule hépatique des mammifères. Arch. d'anatomie microscopique* 19:1. 1923.) in the livers of animals a few hours after a meal. Such an alimentary fatty infiltration disappears after 12 hours. In our mice receiving vitamin E, however, the fatty metamorphosis was permanent and was not due to the consumption of food, because the animals were examined usually more than 12 hours after the last meal.

* Professor D. H. Copeland of this laboratory very kindly performed the necropsies and the histopathological study on some of the rats described in this paper.

DIETETIC HEPATIC INJURIES AND THE MODE OF ACTION OF TOCOPHEROL

By Klaus Schwarz

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In this paper, three different types of fatal liver injuries are to be described which can be produced by dietary means; *i.e.*, by varying a purified regime in several directions, so that conditions of special metabolic stress may arise. These three kinds of dietary liver injury are not only fundamentally different in their causal conditions but they can also be distinguished histologically. Furthermore, they show differences in the way they can be influenced by some liver protective agents, for instance, by sulphur-containing amino acids. The three kinds of liver damage are: (1) the disturbances developing after feeding diets containing a highly purified alkali-treated casein, the so-called casein VI^{1, 2}; (2) the damage produced by feeding diets containing 10 to 20 per cent cod-liver oil³; and (3) the damage which can be produced by using yeast as a main protein source.⁴

It was found that growing rats, under these different conditions, die after some weeks. The disturbance of liver function was the main reason for their death. Each of these three types of liver injuries can be inhibited by vitamin E.

It is now known why, in these experiments, the liver damage was so severe and sometimes appeared before any other sign of vitamin E deficiency could be seen. It is remarkable that calcifications were sometimes found in these livers, especially in smaller lobes. Calcifications also have been described by Fenn and Goetsch⁵ in degenerated muscles. This may indicate that the deviation of biochemical functions in damaged livers is of the same kind as in degenerated muscles. Our diets have been varied in several ways without remarkable effects upon the capacity to induce liver injuries when no protecting agents were given. The only ingredient of the diets which never has been changed is the salt mixture, and experiments in this direction may be of some interest.

In 1940, we intended to find out whether p-amino benzoic acid is a vitamin for rats. Casein has a very high content of this substance,¹ the amount of p-amino benzoic acid found in crude casein being as high as in yeast or liver. It is difficult to prepare casein which is free of this growth-promoting factor. Therefore, as starting material, a highly purified casein from Merck was used. It was found that treatment at pH 8.5 in the heat followed by precipitation with acid results in a product which is practically free of amino-benzoic acid. This product was named casein VI. Young rats on diets containing 15 per cent casein VI died after several weeks. No outstanding external symptoms could be seen. In every case, death occurred following a coma of a few hours duration. Several times it has been possible to induce coma and death in small mice by transferring a filtrate of the blood of dying rats. The dead rats show a striking degeneration of the liver, localized in the central parts of the lobules. The cells in the degenerated

centers are partly, but not too much, infiltrated with fat. Hemorrhages occur. The endothelium of capillaries is free of fat, but shows ceroid pigment.

It may be mentioned that, in about 1,300 rats with liver damages, a typical cirrhosis has never been seen. Possibly this is because the animals did not live long enough.

It ought to be mentioned also that it is important not to give too much vitamin E before beginning the dietary experiment. As a rule, the young rats were put on experimental diets when at 28–30 grams in weight. The mothers and their litters were fed rice and skimmed milk and nothing else, starting from the day of birth.

Casein VI has nearly the same elementary composition and quite the same optical rotation as the starting material. P-amino benzoic acid was completely ineffective regarding the liver damage. Many other substances were tested and also found to be negative. Twenty milligrams of choline chloride were ineffective. Usually, every rat in these experiments received one milligram of choline chloride daily. This was necessary and sufficient to avoid hemorrhagic kidney degenerations.

Sulphur-containing amino acids were without any effect upon the casein VI damage, though it is quite certain that in casein VI cystine is transformed into lanthionine by the alkaline treatment. To make certain that this change is not the reason for the liver injury, purified diets containing a certain amount of lanthionine were fed. In these experiments, no effect of lanthionine upon the livers of growing rats could be seen. Therefore, it seems rather unlikely that the content of lanthionine is the cause of the casein VI damage. It may be mentioned that ethionine also did not induce liver damages.

The fact that sulphur-containing amino acids are not concerned in this liver injury is noteworthy. This is one of the main differences between the casein VI damage and the third type of liver injury to be discussed here, the so-called "rat eclampsia." It is reasonable to conclude that, by the alkaline treatment, another protecting substance in the casein must be split off or destroyed. It has not been possible to demonstrate this factor, although several attempts have been made.

We have found a few substances which are able to inhibit the development of the casein VI injury. Twenty milligrams of xanthine have a protective influence. Xanthine was formerly found by Forbes and McConnell,⁶ Neale and Winter,⁷ and other groups to protect against the liver damage induced by chloroform and similar agents.⁸⁻¹⁰

In the search for factors in natural materials active against casein VI damage, it was found in 1941 that wheat germs had a high capacity for preventing the liver injury. In 1942 to 1943, the active compound of wheat germs was concentrated and nearly isolated, and it was found to be identical with vitamin E. This result was surprising, since all of the animals received 50 micrograms of synthetic dl, α -tocopheryl acetate every week. This seemed to be sufficient to protect young rats against the development of disturbances in the sexual sphere. As a matter of fact, an approximately 17-fold increase in the amount of vitamin E was necessary to protect the

animals against liver damages. They needed 120 to 130 micrograms of synthetic dl, α -tocopheryl acetate daily for full protection. In subsequent experiments, the vitamin E was added to the fat before mixing the diets. Five milligram per cent was sufficient in every case to inhibit liver injuries, though it is quite evident that a part of this amount is destroyed when the components of the diet have been heated during its preparation.

Thus, it has been established that vitamin E has an influence on the functions of the liver. When this effect was found, experiments were started to ascertain whether other liver damages also could be inhibited by vitamin E. In the veterinary literature, a special toxic liver dystrophy had been described as arising in pigs when large doses of cod-liver oil are administered.¹¹⁻¹³ This induced a similar experiment with diets containing normally purified casein combined with relatively large amounts of cod-liver oil. When fed with these diets, young rats showed a severe failure of growth. They were seriously damaged—the muscles getting dystrophic, the backs hunching extremely, and the fur being rough and unkempt. When the amount of cod-liver oil was 20 per cent, all animals died after several weeks. They showed severe alterations of the liver, and, in some cases, but not in all, an evenly distributed degeneration of liver cells was found. The breakdown of liver functions seemed to be the immediate cause of death. The animals showed no body fat at all, while their liver cells were well-filled with big lipid granules. This may indicate, perhaps, a derangement of fat metabolism.

With 5 milligram per cent of synthetic dl, α -tocopheryl acetate, no animal died. The growth of this group was much improved but not quite comparable with the rate of growth in control groups. The animals were sacrificed after 140 days of experiment. No pathological findings except a calcification of the kidneys could be found. The calcification can be traced back to the hypervitaminosis D. It can be reproduced by administration of vitamin D alone without cod-liver oil.

The protective effect of vitamin E upon the cod-liver oil injury in rats is in accordance with the well-known findings of other investigators, especially of Mackenzie,¹⁴ Dam,¹⁵⁻¹⁷ and Mason.¹⁸ The literature about toxic effects of large doses of cod-liver oil is rather complicated and difficult to survey. In part, injuries are described which are typical symptoms of vitamin E deficiency.³

The third dietary liver injury found to be influenced by tocopherol is the damage developed if yeast protein is fed as the main source of protein in synthetic diets. Yeast is very poor in cystine and low in methionine, so that this damage surely is comparable with the well-known findings of Weichselbaum,¹⁹ György,²⁰⁻²² and other groups²³⁻³² working on yeast diets or on low protein diets. The liver injury in these animals begins in the lobular periphery. The cells are filled with fat, as are star-cells. It is remarkable that the kidneys of these animals show a severe glomerulo-nephrosis,³³ a condition very seldom found in rats. A certain percentage of the animals developed convulsions shortly before death occurred.³⁴ It is difficult to observe these convulsions, since most of the animals lapse into coma during the night and are found dead in the morning. The whole syndrome with liver damage, glomerulo-nephrosis, and convulsions was named "rat-eclampsia."

Further investigations are necessary to learn whether the rat-eclampsia may be compared with human eclampsia, and if it can be regarded as a model for studies on problems connected with this group of diseases.

In earlier publications, it was thought that this liver damage was caused only by the lack of sulphur-containing amino acids.²⁸⁻³² This is not the only reason, as has been proved by these experiments and the independent experiments of György.²² The absence of tocopherol is necessary at the same time. Vitamin E alone, or sulphur-containing amino acids alone are able to inhibit the developments of rat-eclampsia. Therefore, it is clear that this disease is not identical with the isolated lack of sulphur-containing amino acids or with the lack of Vitamin E alone. It is no simple combination of two diseases but is a special case involving two quite different dietary components. The term "ambogen" has been introduced for this type of deficiency disease. The existence of an ambogen disease seems to prove a special metabolic correlation between the compounds affected.

Several groups of investigators have not been able to find liver damage on diets low in sulphur-containing amino acids.³⁵⁻³⁷ The explanation for their failure can be found in the amount of tocopherol administered in the diets. The relation between vitamin E supply and prevention of liver injuries has been stressed recently by Himsworth and Lindan.³⁸

The connection between sulphur-containing amino acids and vitamin E seems to be the reason for the protein-sparing effect of tocopherol found by several groups of investigators.³⁹⁻⁴¹ Cystine should be regarded as the limiting factor in diets low in casein.

Recently, experiments were started which combined yeast protein with 20 per cent cod-liver oil. In the first group of animals on this diet, death occurred before liver lesions could develop. The rats were in a severely damaged condition, and, when they died, a new syndrome was seen. The lungs were infiltrated and the hearts were pale and seemed, to the naked eye, to be degenerated. There were impressive hydrothoraces and hydropericardia. The histological examination, however, which has not yet been completed, demonstrated that only small parts of the heart muscle really were degenerated. The fluid in the lungs is not an acute edema but seems to be infiltrated through the pleura. A pleuritis and pericarditis with fibrinosis are found. Further investigations must be made before it can be established whether or not an infection is affiliated with these changes. At any rate, it is remarkable that 5 milligram per cent of dl, α -tocopheryl acetate was able to protect all the control animals against this disease.

In summary, it should be stated that these results demonstrate that tocopherol is important for the liver and that, in its functions, it is closely correlated to several other agents, especially to sulphur-containing amino acids, to xanthin, and to unsaturated compounds in fat. These relations are complicated and must be elucidated by subsequent experiments of a quantitative nature. This may, perhaps, help in understanding the versatility of the symptoms of vitamin E deficiency under different conditions.

It is impressive that 1 mol of tocopherol has the same effect in preventing "rat eclampsia" as 200-400 mols of cystine or methionine. This may, *perhaps*, indicate that vitamin E has a catalytic function, where cystine, or

compound derivatives are used as substrates. It is quite certain, for chemical reasons, that tocopherol itself cannot participate directly in transmethylation, but it may be possible that the function of tocopherol is, in some way, affiliated with transmethylation steps. Thus, vitamin E would be important for fat metabolism and for sulphur-containing amino acids at the same time.

Attempts have been made to discover the effects of tocopherol-therapy in cases of human liver diseases. The influence upon epidemic hepatitis is difficult to demonstrate because this disease has a ready tendency for recovery. A certain percentage of the cases do not respond immediately to vitamin E therapy, while others seem to be influenced. Though the results look favorable generally, further careful experiments are necessary. The application of tocopherol in cases of liver damage is complicated if the bile-flow is reduced or stopped. A vicious cycle exists. Insufficient absorption of vitamin E permits further injury to the liver, and this reversibly reduces the bile-flow and tocopherol-absorption, *et cetera*.

In order to break this cycle, it is necessary to give water-soluble vitamin E preparations or to inject vitamin E in oil solution intramuscularly. These injections are not well absorbed, and it is not possible to estimate the amount of tocopherol which actually reaches the liver. It will, perhaps, be possible to find special water-soluble vitamin E compounds which can be hydrolyzed specifically in the liver. It was found that rat liver is rather active in hydrolyzing tocopherol-phosphate.

When large doses of dl, α -tocopheryl acetate were administered to cases of jaundice due to occlusion of the bile ducts, and impressive reduction of bilirubin in blood occurred. This treatment may obtain a certain value in preparing patients for operations. In about 30 per cent of these cases, bilirubin did not react. In the urine of others, but not in every case, a colorless substance was detected which can be oxidized to a dark green pigment, thus disturbing the Gmelin reaction for bilirubin.

Many more experiences must be collected before final judgment of the value of vitamin E therapy in human liver injuries can be given. In order to avoid deprecation of the real value of vitamin E therapy, one should be careful and not be too optimistic. Perhaps, it will be favorable and necessary to combine vitamin E with other liver-protecting agents.

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Discussion of the Paper

DR. N. S. SCRIMSHAW (*Department of Obstetrics and Gynecology, University of Rochester, School of Medicine and Dentistry, Rochester, New York*): In Dr. Schwarz's generally excellent presentation, I must object to his use of the term "rat eclampsia." He feels justified because he has used it to describe a condition of sudden onset, characterized by kidney and liver pathology and convulsions. However: (1) The renal and hepatic lesions of human eclampsia are not generally regarded as specific, and they do differ from those described by Dr. Schwarz in his rats. (2) Convulsions occur in both man and experimental animals for a very wide variety of causes and are not in themselves suggestive of eclampsia. (3) It seems most unwise to confuse the literature by applying the term "eclampsia" to a condition which not only is not comparable to human eclampsia in any specific fashion, but which also is not associated with pregnancy. None of the animals in which Dr. Schwarz described "rat eclampsia" was pregnant. (4) The term "eclampsia-like syndrome" has already been guardedly applied to several experimentally induced conditions in experimental animals, including the rat. In none of these was there definite assurance that the syndrome was really comparable to human eclampsia.

I do not intend this specific criticism to detract from the very challenging suggestions which Dr. Schwarz has advanced, particularly in regard to the efficacy of alpha-tocopherol therapy in human infectious hepatitis and obstructive jaundice. He has been modest in his presentation, for, in private conversations, he revealed that he has had the opportunity to make very extensive clinical trials and objective evaluations of the drop in serum bilirubin in a large number of cases.

This is certainly work which deserves to be seriously considered and carefully repeated in this country.

TOCOPHEROL AND HEMOLYSIS *IN VIVO* AND *IN VITRO*

By Paul György and Catharine S. Rose

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Work of Houssay and Martinez,¹ published in 1947, suggested that the same mechanisms might be active in protecting the rat against the toxic effects of alloxan that had been found of value in protection of the liver against acute massive necrosis induced by dietary means. Among other effects of dietary factors, they observed longer survival after alloxan injection in rats which had received methionine (but not choline) as a supplement to a diet high in lard, or had been given a diet in which the lard was replaced by vegetable oils. It has recently been shown that tocopherol is a protective agent for the liver, and experiments were undertaken to determine whether the advantage of vegetable fats might depend on their content of tocopherol.

The rapid hemolysis of the blood of tocopherol-deficient rats, which was the major finding of this investigation, was observed first at autopsy.² The kidneys of rats which died a few hours after injection with 160 mg. per kg. of alloxan intraperitoneally were completely engorged with blood. Hemoglobinuria and hemoglobinemia were then followed, and the extent of hemolysis in some cases was astounding. Within 10 minutes after the injection, the centrifuged hematocrit tube showed the serum layer dark red in color, and, in about half an hour, the layer of cells, instead of being about 50 per cent of the total volume, was almost undetectable. In those animals which survived, the hemoglobin was excreted and there was no further hemolysis.

TABLE 1 shows the effect of variations in the tocopherol-deficient diet

TABLE 1
EFFECT OF DIETARY FACTORS ON EARLY MORTALITY AND HEMOGLOBINURIA FOLLOWING
ADMINISTRATION OF ALLOXAN

<i>Diet</i>	<i>Rats dead in first two days (%)</i>	<i>Hemoglobinuria (%)</i>
High lard	55	100
High lard with tocopherol	25	0
High lard with yeast	15	60
High lard with yeast and tocopherol	0	0
High vegetable shortening ("Vream")	10	0
High "Vream" with yeast	15	0
Low fat	25	60
Low fat with tocopherol	0	0
Low fat with yeast	5	55
Low fat with yeast and tocopherol	5	0

and of supplementation with tocopherol on hemolysis. The diets used have been described previously.² The high fat diets contained 38 per cent of the lard or vegetable shortening. Animals in the low fat groups received 3 drops of corn oil daily and 3 drops of percomorph oil weekly as the only

fat in their ration. In the yeast groups, 5 per cent of yeast replaced an equal amount of carbohydrate. The tocopherol-supplemented animals received 3 mg. of mixed natural tocopherols daily. The rats, young females weighing 100 to 120 grams, were given the experimental ration for one month before being injected with alloxan.

With the low-tocopherol diets, the degree of hemolysis varied. There was an advantage of a low content of fat and, in some groups, benefit from yeast was observed. Tocopherol, whether it was given as a natural constituent of the dietary fat or as a separate supplement, gave complete protection in all cases, without a single exception. There was mortality in almost all groups during the first 2 days after alloxan injection. Tocopherol reduced the incidence of mortality, particularly in the high-fat groups, apparently by prevention of extensive red blood-cell destruction with the consequent blocking of the kidney.

To return to the original question: Would tocopherol protect the islets of the pancreas against destruction by alloxan? The data gave no support to this hypothesis. Diabetes, as measured by blood sugars taken 48 hours after injection of alloxan, was equally severe whether or not tocopherol was given (TABLE 2). Blood-non-protein nitrogen determinations indicated that

TABLE 2
EFFECT OF DIETARY FACTORS ON DIABETES, KIDNEY DAMAGE, AND SURVIVAL FOLLOWING ADMINISTRATION OF ALLOXAN

<i>Diet</i>	<i>Blood sugar 48 hours (mg. %)</i>	<i>Blood NPN 48 hours (mg. %)</i>	<i>Survival 7 days (%)</i>
High lard	500	200	5
High lard with tocopherol	490	90	15
High "Vream"	510	110	35
Low fat	660	170	45
Low fat with tocopherol	520	105	70

kidney damage might be somewhat more serious in the tocopherol-deficient animals, but histological examination showed the same type of injury in all cases. The increased nitrogen retention in the deficient groups was probably a result of the blocking of the kidney with blood. Equally, tocopherol showed no effect in prolonging the life of the rats beyond the two-day period already mentioned. On the basis of our experiments, the protective effect of tocopherol is limited to the red blood cell. It gave *no* protection against the development of alloxan diabetes.

Hemolysis has not been mentioned in connection with alloxan effects in rats but has been observed in rabbits, as reported a few years ago by Kennedy and Lukens³ and within the past year by Gualandi and Campana.⁴ These animals were presumably normal and there is no information about their vitamin E status.

Our further experiments were directed towards an explanation of the hemolysis and the way in which tocopherol protected against it. If one attributes any effect to alloxan itself, it must be one which occurs very

rapidly, since alloxan disappears from the blood stream within 2 or 3 minutes after intravenous injection. The main reactions of alloxan with which we must be concerned are shown in FIGURE 1. In neutral solution, alloxan

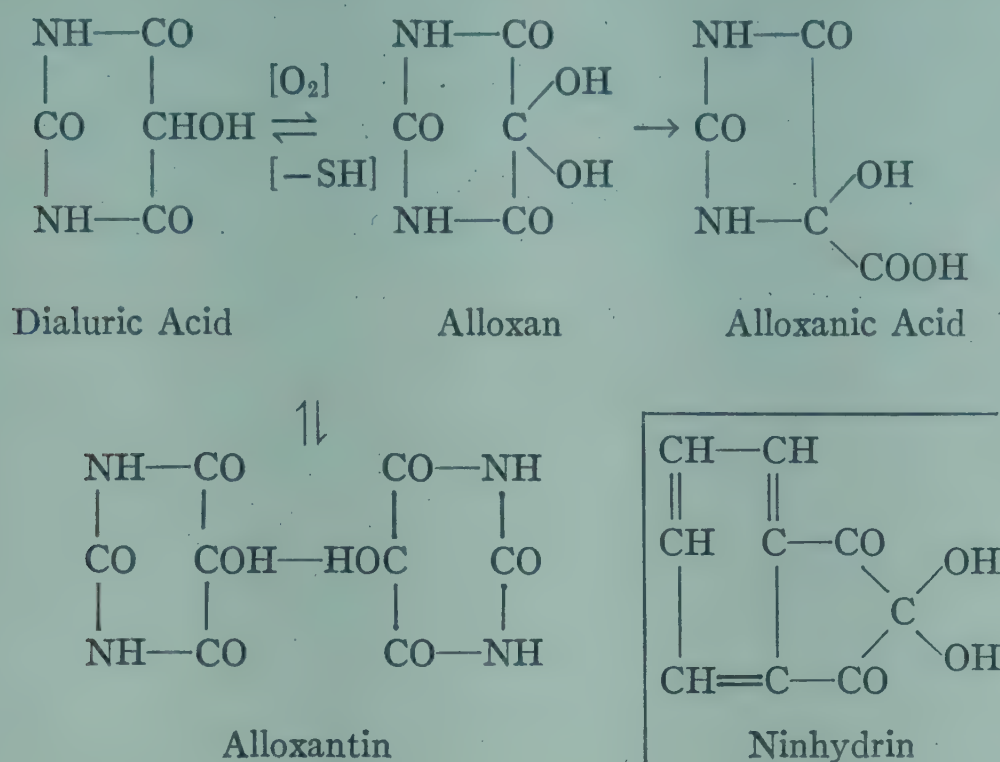


FIGURE 1.

is converted very rapidly to alloxanic acid by molecular rearrangement. Under physiological conditions this reaction is not reversible. In the presence of such mild reducing agents as the sulfhydryl compounds, alloxan is reduced to dialuric acid. With less than equivalent amounts of the reducing agent, an intermediate compound, alloxantin, which may be considered as a double molecule consisting of one molecule of alloxan and one molecule of dialuric acid, is formed. In dilute solution, alloxantin is almost completely dissociated into alloxan and dialuric acid. This system is readily reversible, dialuric acid being oxidized by molecular oxygen. In the presence of ammonia, alloxantin will form murexide, the ammonium salt of purpuric acid. Ninhydrin, the formula of which is given for comparison with that of alloxan, was also considered as a possible hemolyzing agent, since, as far as the triketone portion of the molecule is concerned, it is analagous to alloxan and forms a similar series of compounds.

A number of these compounds were tested *in vivo* on rats which had been kept for one month on a tocopherol-deficient ration. Alloxanic acid was without hemolyzing effect. Alloxantin showed about the same activity as alloxan, while dialuric acid proved to be about twice as effective. Ninhydrin was by far the most active material tested, giving extensive hemolysis at a level of 25 mg. per kg., about $\frac{1}{6}$ the required amount of alloxan. With 10 mg. per kg. of ninhydrin there was no hemolysis.

None of these substances caused any hemolysis of the blood of animals receiving supplements of tocopherol. Since ninhydrin was effective at such a low level, it seemed useful to see how high one would have to go to overcome the tocopherol effect. Ninhydrin is more toxic than alloxan and animals receiving over about 80 mg. per kg. do not survive. This did not

affect the experiment, however, since the hemolytic reaction is very fast. We continued to raise the dose in tocopherol-treated rats, giving 100, 200, and finally 500 mg. per kg. Two animals received this highest dose. One survived only 15 minutes, but the other lived for 1-½ hours. In neither was there any trace of hemolysis.

Simultaneously with these tests, we had been trying out the possibility of producing hemolysis *in vitro*. It was found that under appropriate conditions the red blood cells of animals deficient in tocopherol could be completely hemolyzed, while those of tocopherol-treated animals were never affected. Blood was collected from the tail of the rat into a tube containing normal saline and sodium citrate. The sample was centrifuged, washed, and made up in a 5 per cent suspension with saline. One volume of this suspension was mixed with one volume of phosphate buffer (.05 M monopotassium phosphate and .039 M sodium hydroxide; pH 7.4) containing the material to be tested, the tube was incubated at 37°C. for 15 minutes, and the progress of hemolysis was followed by observation of the decrease in opacity of the suspension.

A comparison of the results obtained *in vitro* with those previously found *in vivo* is given in TABLE 3. Alloxan did not cause hemolysis, nor did nin-

TABLE 3

HEMOLYSIS BY ALLOXAN AND RELATED COMPOUNDS IN TOCOPHEROL-DEFICIENT RATS

Compound	Hemolysis	
	<i>in vivo</i>	<i>in vitro</i>
Alloxan	+	—
Alloxantin	+	++
Dialuric acid	++	++++
Ninhydrin	++++	—

hydrin, which is comparable in structure. Under the conditions used, dialuric acid in a concentration of 0.7 millimols per liter of the mixture caused complete hemolysis in 15 to 30 minutes. There was hemolysis with alloxantin, but not so rapidly as with dialuric acid, which suggests that the results obtained with dialuric acid did not depend on the formation of alloxantin in the mixture and, rather, that the effect of alloxantin was due to the dialuric acid moiety. In both methods of testing, dialuric acid stands out as the active member of the system of compounds related to alloxan.

These experiments have answered the first question which arose in connection with tocopherol: whether its action was in the body fluids or in the red blood cell. Since washed red blood cells of animals which had received tocopherol were resistant to the hemolytic action of dialuric acid, the protection was a function of the cell itself. Another question was whether tocopherol was acting as such in the cell, or whether, under its influence, a more resistant cell was manufactured. This was tested by addition of synthetic tocopherol to the reaction mixture. Tween 80 was used to get the tocopherol into solution. Four grams of Tween were mixed with one

gram of tocopherol and the solution diluted 25,000 times with phosphate buffer. Control tests with Tween alone showed that it had no effect on hemolysis in the dilutions used. In the first experiments, the tocopherol was added to the suspension of the red cells of a tocopherol-deficient animal just before the dialuric acid. There was very definite protection. With a dialuric-acid concentration of 0.7 millimols per liter, tocopherol in a concentration of 0.009 millimols per liter or a ratio of about 1 mol to 80 mols of dialuric acid prevented hemolysis completely. To determine whether the added tocopherol was acting in the solution or in the cell, red blood cells were incubated with tocopherol for half an hour at 37°C., centrifuged, the supernatant fluid removed, and the cells resuspended in saline and treated with dialuric acid. When this procedure was followed the activity of the tocopherol was increased tenfold.

In these experiments, the requirement of tocopherol seemed to be more closely related to the concentration of cells than to the concentration of dialuric acid. The 0.009 mM. per liter of tocopherol protected equally against the hemolyzing effect of 0.7 and 1.4 mM. per liter of dialuric acid, but, with either concentration of dialuric acid, gave no protection when a 10 per cent suspension of red blood cells was used in place of the usual 5 per cent suspension.

Since the red blood cells were able to adsorb tocopherol from the surrounding medium, the cells of tocopherol-deficient rats were incubated with the plasma of tocopherol-treated animals, which, by calculation, contained enough tocopherol to afford complete protection. Not only was the plasma itself quite ineffective, it inhibited the effect of added tocopherol. It may be assumed that the added tocopherol was bound by the proteins of the plasma.

One cannot explain the effect of tocopherol as a reaction between it and dialuric acid. It is a mild reducing agent, but rather strenuous methods are necessary for reduction of dialuric acid. Tocopherol is most frequently considered as an antioxidant, and dialuric acid is readily autoxidizable. It is likely that it is some molecule or radical formed during the oxidation of dialuric acid that is the actual hemolyzing agent and which reacts with tocopherol.

It has been shown that alloxan is reduced by sulfhydryl compounds in the body, so alloxan and cysteine were tested together *in vitro*. The combination was found to behave like dialuric acid. Using 0.2 mg. of alloxan per ml. and varying amounts of cysteine, hemolysis was found to occur with as little as 0.01 mg. of cysteine per ml. This makes the system even more active than dialuric acid and supports the idea that a reactive intermediate is responsible for the hemolysis.

It was interesting to find that the three compounds used to reduce alloxan would themselves cause hemolysis. This reaction was slower than that with dialuric acid, never appearing in less than 2 hours, and occurred only with considerably larger amounts of reagent. The reaction resembled that of dialuric acid, however, in occurring only with cells deficient in tocopherol. These compounds are all autoxidizable. Ascorbic acid and dehydroascorbic acid are indeed analogous in structure to dialuric acid and alloxan. Cysteine

was the most active of the three compounds. On a molar basis, it was two to three times as effective as ascorbic acid and five times as effective as glutathione. Typical results obtained with cysteine are shown in TABLE 4.

TABLE 4
HEMOLYSIS *In Vitro* WITH CYSTEINE

Cysteine in mol./liter	Degree of Hemolysis		
	3	7	18 hours
5.2	—	—	++++
2.6	±	+	+++
1.3	+	++	Complete
0.65	—	—	++

There was most rapid hemolysis with 1.3 millimols per liter of cysteine, the rate decreasing with both higher and lower concentrations. The decrease in hemolysis at higher concentrations is typical of hemolyzing agents and is a result of a protective layer formed around the damaged cell. This was observed also with dialuric acid. The rate of hemolysis began to drop off above 0.2 mg. per cc., and with 0.75 mg. per cc. there was no hemolysis. That hemolysis was always delayed with cysteine, glutathione, and ascorbic acid may be due in part to overlapping of the hemolytic and protective levels.

The *in vitro* procedure has proved to be a simple and convenient method of studying some aspects of tocopherol metabolism.

The animals used in these studies were obtained from Sprague-Dawley and kept in our laboratory usually from 1 to 3 weeks before being put on experiment. The injection experiments, as well as the tests on washed red blood cells, had shown that when these animals had received a tocopherol-deficient diet for only one month all of their red blood cells could be hemolyzed. The blood of normal animals was not hemolyzed by dialuric acid *in vitro*. If the rats were fasted 48 hours, however, a procedure frequently followed when alloxan is used to produce diabetes, there was a slight degree of hemolysis, although probably not enough to have caused serious complication if alloxan was injected. When rats were tested at various intervals after being transferred to the tocopherol-deficient diet, in only 3 to 7 days the red cells showed sensitivity to dialuric acid which might be estimated as ++ on the usual scale of + to ++++, and after about 2 weeks showed the maximum rate of hemolysis. In contrast to these animals, a group may be mentioned which had received a supplement of 3 mg. of mixed tocopherols daily for a month and a half. After 3 months on the deficient ration without supplement, the blood of these animals was still almost completely resistant to hemolysis.

A confirmation of the fact that our "normal" animals bore a much closer resemblance to the deficient ones than to those receiving a generous allowance of tocopherol was found in the levels of plasma tocopherol. Treated animals had an average value of 1.0 mg. per cent, the stock rats about $\frac{1}{3}$

of this amount, 0.38 mg. per cent and the rats on the deficient diet, 0.27 mg. per cent.

Tests have been made to determine the amount of tocopherol necessary to protect the rats against hemolysis. Animals weighing 100 to 120 grams were placed on the high lard ration and given daily supplements of 0.1, 0.2, and 0.4 mg. of mixed natural tocopherols. After one week, only those receiving the highest dose were completely protected. Only a few animals have been tested at intermediate levels and no more precise definition of the protective dose can yet be given.

The rapid adsorption of tocopherol observed *in vitro* has been borne out by tests *in vivo*. A single dose of 1.5 mg. of tocopherol given orally to deficient rats gave complete protection in 24 hours. The protective effect did not disappear completely for about 10 days. Feeding of $\frac{1}{2}$ mg. of tocopherol for 3 consecutive days did not give as good protection as the single large dose. The tests with plasma *in vitro* have indicated that the process of tocopherol utilization in the living animal is not so simple as the tests in the synthetic medium might have suggested.

The low level of tocopherol in the newborn has been the subject of a number of recent investigations.⁵ We have studied the blood of young rats and found that the cells of rats 1 or 2 hours old showed almost the maximum degree of hemolysis by dialuric acid. Litter-mates whose blood was tested the next day were already completely protected.

It is hoped that more extensive studies of this type may be made. The method gives an opportunity of studying a physiological activity of tocopherol *in vitro*. A comparison of different tocopherols would be of interest in this connection. Symptoms of deficiency, according to this classification, appear when the rat has been on a tocopherol-deficient diet for only a few days or weeks, and rigid exclusion of tocopherol from the diet for such a long period of time as is necessary to produce most of the physiological manifestations of deficiency in the rat is not required. The simplicity of the procedure would make it most useful as a method of bioassay for vitamin E. The measurements of protective and curative doses which have been discussed illustrate how such an assay might be carried out.

The fact that tocopherol is present in the erythrocyte to protect it against the effect of alloxan furnishes a teleological basis for an argument that it is there for that purpose. That hemolysis can be caused by cysteine, glutathione, and ascorbic acid, which are normal metabolites, makes this more probable. On the other hand, there is apparently no great need for the system under ordinary circumstances. Destruction of red blood cells has not been a characteristic feature of vitamin E deficiency. Our rats showed no signs of anemia before they were injected with alloxan. This defense may be of importance only under abnormal conditions of metabolism. The human adult is not likely to suffer from this deficiency except in such special cases as conditions involving failure of fat absorption. The experiment with the young rats suggests that the human fetus may be in a peculiarly defenseless position against such hemolyzing agents as have been discussed.

In summary, hemolysis caused by alloxan and related compounds seems

to be linked with the reversible oxidation-reduction relation between alloxan and dialuric acid, some intermediate of the reaction probably being the actual hemolyzing agent. Cysteine, glutathione, and ascorbic acid resemble dialuric acid in being autoxidizable and probably act in the same fashion in causing hemolysis. The protective action of tocopherol is within the red blood cell and may best be explained as an antioxidant effect.

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Discussion of the Paper

DR. P. GYÖRGY (*Department of Pediatrics, School of Medicine, University of Pennsylvania, Philadelphia, Pa.*): The hemolysis demonstrable in rats with an average body weight of 100–150 Gm., only three to seven days after the animals were put on a tocopherol-free diet, appears to indicate an insufficient, or at least sub-optimal, intake of vitamin E by these animals while fed a regular stock diet. The hemolysis test may be applied for bio-assay of vitamin E, requiring a much shorter preparatory period and a simpler technique than the usual resorption test.

Possible practical implications may be based on the following observations and its extrapolation to conditions in man: The newborn litter of rats kept on a normal stock diet (mixed grains and greens) shows positive hemolysis test, whereas, simultaneously, the red blood cells of the mother animal exhibit no hemolysis under the influence of dialuric acid. Thus, it may be assumed that the mother animal is sufficiently supplied with vitamin E and the newborn litter is deficient in vitamin E. Six to twelve hours after birth, the hemolysis test becomes negative in the newborn animals, probably under the influence of the colostrum, which is rich in vitamin E. These findings seem to support the view that either the transfer of vitamin E through the placenta to the fetus is limited or the metabolism of vitamin E in the fetus requires a higher supply than furnished by the regular stock diet of the mother animal.

Assuming that similar conclusions may be applied to man, the observation of extra-medullary hematopoiesis and erythroblastosis as—after Miller—the most specific finding in the newborn infants of diabetic and pre-diabetic mothers is of special interest. Such a finding should be the result of increased destruction of red blood cells, which in turn indicates exposure to some hematotoxic agent. The question arises whether such an agent could be similar in its action to alloxan, producing slow and continuous hemolysis in the vitamin E-deficient fetus and leaving intact the red blood cells of the mother, with her sufficient stock of vitamin E. The origin of this hemolyzing, perhaps alloxan-like, agent is probably the maternal metabolism. The agent may freely invade the circulation of the fetus, where its hemolyzing effect will become evident owing to the absence of

sufficient amount of protective vitamin E. The absence of anemia and the lack of its progression after birth may be explainable on the basis of a relatively low concentration of the toxic substance in the blood before birth, and its complete disappearance—by cutting off its source—after birth.

This theoretical consideration is open to exact critical study and is presented here only as a remote, but not entirely improbable possibility.

In Rh-incompatibility, several research workers, among them Sir Leonard Parsons (Birmingham, England), N. Philpott (Montreal), and the speaker have discussed the rôle of liver injury as a central pathogenetic factor in the outcome of the disease, including especially the hemorrhagic manifestations and also kernicterus. Hepatic injury follows some as yet unidentified phases of the antigen-antibody reaction, which in itself occurs obviously as the first chain in the events linked with the syndrome of Rh-incompatibility. The hepatic injury is characterized chiefly by acute zonal or massive necrosis. Protection of the liver may be attempted by methionine. Philpott has already presented preliminary evidence in favor of this view. We may now add the further possibility that during the sensitization-process, which is at the very bottom of Rh-incompatibility, the normally low vitamin E stores of the fetus and the newborn are further depleted. Hepatic necrosis is one of the characteristic sequelae of vitamin E deficiency under particular conditions (see also the papers of Schwarz and Hove in this monograph and the relevant studies of the speaker). Edema and pulmonary hemorrhage are often encountered in very severe human erythroblastosis (*hydrops fetalis*) and seen also in experimental vitamin E deficiency in animals. The postulated but unproven vitamin E deficiency in human erythroblastosis is independent from the immunological antibody-antigen reaction of Rh-incompatibility. Studies are in progress jointly with Dr. Carl Bachman, Professor of Obstetrics at the School of Medicine, University of Pennsylvania, on the combined use of methionine and vitamin E in the prevention of the unspecific sequelae of Rh-incompatibility, such as hepatic necrosis, kernicterus, pulmonary hemorrhage, and edema. It is *not* expected that the anemia, based on the immunological antigen-antibody reaction, will be influenced by the administration of methionine and vitamin E.

IV

PRACTICAL NUTRITIONAL ASPECTS OF VITAMIN E INTRODUCTORY REMARKS

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“Practical nutritional aspects” of vitamin E implies a quantitative knowledge and a quantitative application of vitamin E in the nutrition of farm animals and of man. The 1939 Conference had no session on practical nutritional aspects because at that time the vitamin E field was still in its “qualitative phase.” Since then, foods and feeds, body-tissues and excretions have been analyzed for vitamin E. Losses of vitamin E due to food processing have been studied. Increased requirements induced by physiological stresses have been established. Also, methods for the determination of vitamin E concentration in blood have been developed and applied to a variety of metabolic studies. In effect, the “quantitative phase” of vitamin E research has been entered.

One practical outcome which has already emerged resulted from the discovery that the process of aging flour with NCl_3 destroyed 80 per cent of the vitamin E.¹ Because of this loss of vitamin E, chemical treatment of flour was discontinued in Germany five years ago. Another practical advance is the adoption of regulations in Canada covering the use of vitamin E in food and pharmaceuticals. The Canadian Food & Drug Division has ruled that the amount of vitamin E in a preparation labeled to contain this vitamin must furnish at least 10 I.U. in a minimum daily dose. When the amount is over 50 I.U. in a daily dose, the preparation is considered therapeutic in nature and must be so labeled.²

Consequently, it seems that the basic purpose of the papers to be presented in this section of the monograph is to evaluate critically the few data in the literature which bear on the human and animal requirement for vitamin E and to contribute new data from controlled and scientifically designed experiments which will lead us nearer to the answer of how much vitamin E each individual, animal and human, needs for optimum nutrition.

Somewhat pertinent to this problem, we attempted several years ago to extrapolate from the vitamin E needs of the rat to a value for the minimum daily requirement of humans.³ On the basis of direct proportionality to body weight, a requirement for a 70 kg. human would be 60 mg. or more of tocopherol per day. This seemed unreasonably large, so we then tried to relate requirement to quantity of food ingested. Here, however, the value arrived at for humans was only about 12 mg. of tocopherol daily. Thus, although one value seemed much too high and the other much too low, we used the range 12 to 60 mg. of natural tocopherols as the limits within which the daily requirement for vitamin E probably falls.

Recently, Brody, of the University of Missouri, has presented a great deal of evidence showing that various functions and reactions of the body are related not to body weight but to the 0.7 power of body weight.⁴ This function of body weight ($\text{weight}^{0.7}$) has been designated “physiological

weight," in contrast to weight,^{1.0} which is "physical" or "gravitational" weight. Basal energy metabolism, endogenous nitrogen excretion, milk energy production, egg energy production, and many related processes all vary as the 0.7 power of body weight. Consequently, we wondered if vitamin E requirement also varied with physiological, instead of physical, body weight.

We therefore collected the data shown in FIGURE 1, which relates the

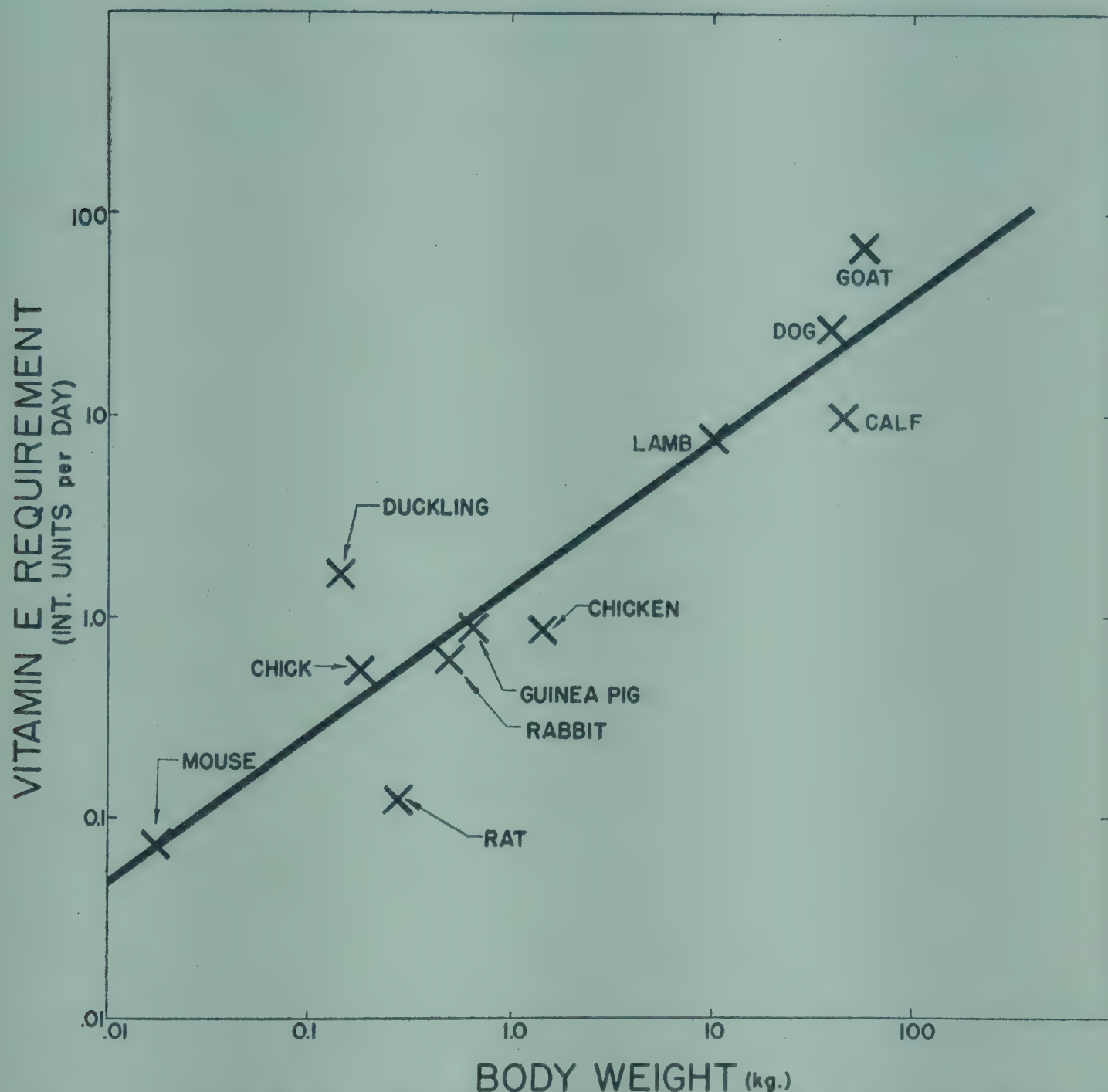


FIGURE 1. Showing the relationship between vitamin E requirement of various species of animal and body weight. The line which best fits the data points has a slope of 0.73, indicating that vitamin E requirement varies with $Wt^{0.73}$. The points from which the straight line was established were obtained by evaluating data reported in the literature for mice,^{5, 6} rats,^{6, 7, 8, 9, 10} chicks,^{11, 12, 13} ducklings,¹⁴ guinea pigs,¹⁵ chickens,¹⁶ lambs,¹⁷ dogs,¹⁸ calves,¹⁹ rabbits,^{20, 21, 22} and goats.^{23, 24}

log. of vitamin E requirement of animal species, where this can be calculated, to the log. of body weight. The straight line which best fits the points in FIGURE 1 has a slope of 0.73, not significantly different from Brody's value of 0.7. This means that for every 100 per cent increase in body weight, vitamin E requirement would be increased by 70 to 73 per cent. For a 70-kilogram human, the value is approximately 30 I.U. of vitamin E per day.

These relationships, vitamin E requirement as a function of physiological weight and the estimated human requirement for this vitamin, are presented merely as working hypotheses to be modified, replaced, or confirmed. The papers which follow will contribute data relative not only to these hypotheses but to a variety of other quantitative aspects of the practical use of vitamin E in nutrition.

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VITAMIN E REQUIREMENT AND ECONOMY IN FARM ANIMALS

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Vitamin E has been shown to be essential in the diet of cattle,¹ sheep,² and poultry.⁶ Attempts to produce the deficiency in pigs³ have been unsuccessful, and the problem has not been studied in other farm animals.

Symptoms of Vitamin E Deficiency

In all herbivorous animals, vitamin E deficiency appears to cause muscular dystrophy. In lambs, the dystrophy occurs primarily in the skeletal muscles and less frequently in the heart.⁴ The heart muscle is more often affected in mature cattle¹ and calves.⁵ Similar muscular lesions are produced by feeding cod-liver oil⁷ in sheep, goats, guinea pigs, and rabbits, but apparently not in calves.⁹ In rabbits, these lesions are prevented or cured by alpha-tocopherol.⁸ Because vitamin E was first demonstrated to be necessary for reproduction in rats, numerous attempts have been made to establish its essential nature in reproduction for goats and sheep,¹⁰ cattle,¹² and swine,³ all with negative results. Other studies have failed to show any improvement in reproductive efficiency of farm animals when vitamin E supplements were added to natural rations.^{11, 12, 13} However, vitamin E in the rations of farm animals appears to have other important functions.

Vitamin E deficiency can be produced consistently in lambs by feeding the ewes a ration of alfalfa hay and beans. Reproductive efficiency is normal, but when the suckling lambs are 2 to 6 weeks of age they manifest muscular stiffness of varying severity. Lambs showing mild stiffness often recover spontaneously, but in the severe cases death losses are the rule unless alpha-tocopherol therapy is practiced. On post-mortem examination, the "stiff" lambs show typical dystrophic lesions of the skeletal muscles.⁴ Only rarely is the heart muscle affected. The dystrophic lambs show changes in the excretion of creatine¹⁴ similar to that of the laboratory animals.¹⁵

The studies of Gullickson *et al.*¹ have shown that tocopherol is a dietary essential for cattle. The deficiency manifests itself by heart failure. Their data fail to show any influence of the deficiency upon reproductive performance. Muscle dystrophy, "white muscle disease," has been reported in calves.¹⁶ This condition is presumed to be a tocopherol deficiency, but conclusive data have not been published. Tunncliffe, of the Montana Station, reports that increasing numbers of calves have exhibited muscular dystrophy during recent years. In calves, the heart muscle is very often the vitally affected organ.⁵

By the use of chemical procedures worked out by Quaife and associates at Distillation Products, Inc., recent studies have been made of the tocopherol content of the blood plasma of ewes and lambs and of the colostrum and milk of ewes fed the dystrophy-producing ration and of other ewes fed a ration of mixed hay, corn silage, and cereal grains.

The deficient ewes (TABLE 1) had an average tocopherol content only 15 per cent as much in their blood plasma, 48 per cent as much in their colos-

TABLE 1
TOCOPHEROL CONTENT OF PLASMA AND MILK OF SHEEP AS INFLUENCED BY DIET

<i>Identification</i>	<i>Ration</i>	
	<i>Deficient</i>	<i>Normal</i>
	$\mu\text{g./100 ml.}$	$\mu\text{g./100 ml.}$
Plasma-ewes	34 \pm 14	251 \pm 45
Plasma-lambs	48 \pm 23	118 \pm 43
Colostrum	591 \pm 333	1334 \pm 461
Milk	60 \pm 28	164 \pm 56

trum, and 38 per cent as much in their milk as the normal ewes. The plasma of lambs from ewes on the deficient ration contained only 42 per cent as much tocopherol as the plasma of lambs from ewes on the normal ration. These differences in the body stores of the lambs and in the content of their mothers' milk would appear to explain why one group of lambs develops muscle dystrophy while the other remains healthy. In this connection, it is interesting to note that the rate of growth appears to influence the onset of the deficiency, since, on the same treatment, rapidly growing lambs manifest dystrophy, whereas twin lambs or those that grow at a less rapid rate seldom show stiffness. It has not yet been possible to measure the content of the various tocopherols in the feedstuffs involved, but the total tocopherol content of the two rations compared was approximately the same. Perhaps the proportion of alpha-tocopherol to total tocopherols was less for the legumes, alfalfa, and beans than for the non-legume feeds fed to the other ewes.¹⁴

Studies on Vitamin E Metabolism

To obtain further data on tocopherol metabolism, a study was made of the placental and mammary transfer of tocopherol in sheep, goats, and pigs. Ewes, does, and sows in gestation were fed a basal ration of standard feeds or the basal ration plus 80 mg. of tocopherols daily per 100 pounds of body weight. Plasma samples from newborn lambs and kids taken before they had suckled (TABLE 2) contained four times as much tocopherol when the dams were supplemented as on the basal ration. Plasma from newborn pigs showed no increase in tocopherols. In all three species, the colostrum from supplemented animals contained two to three times as much tocopherol as the normally fed animals. These data make it quite clear that tocopherols pass the placental membranes and mammary gland in these animals and that the onset of gross deficiency in the young will depend upon the diet of the dam, which in turn controls the body stores of the newborn young and the amounts which it obtains from the milk.

Parrish *et al.*¹⁷ have shown that supplementing the ration of dairy cows with 500 to 1000 mg. of tocopherols daily during the last four weeks of

TABLE 2
TOCOPHEROL CONTENT OF BLOOD PLASMA AND COLOSTRUM

Samples	Dietary supplement	
	None	Tocopherols
Plasma of newborn animals ($\mu\text{g.}/100\text{ ml.}$)		
Lambs	20	94
Kids	16	65
Pigs	120	101
Colostrum ($\mu\text{g.}/\text{gm. fat}$)		
Ewes	47	78
Does	59	154
Sows	186	399

pregnancy increased the tocopherol content of the colostrum from 107 to 150 $\mu\text{g.}$ per gram of fat and that feeding 10 gm. of tocopherols daily increased the colostral content to 487 $\mu\text{g.}$ per gram of fat.

In other experiments, measurements were made to determine the influence of tocopherols at this level upon the utilization of vitamin A. In certain comparisons (TABLE 3) with lambs and kids, feeding 80 mg. of tocopherols

TABLE 3
EFFECT OF TOCOPHEROLS ON THE NEONATAL LIVER STORES OF VITAMIN A ($\mu\text{g.}/\text{gm.}$)

Supplements	Lambs	Kids	Pigs
None	0.19	0.30	5.08
Vitamin E	0.43	0.27	5.26
Vitamin A	0.58	1.14	26.26
Vitamin A + E	0.57	2.52	18.22

per 100 pounds body weight daily to pregnant females during gestation appeared to increase the liver stores of vitamin A of newborn animals, but the effects were not consistent. In these studies, feeding supplemental vitamin E to pregnant sows did not increase the neonatal liver stores of vitamin A in pigs. All of these studies with farm animals are handicapped by the lack of simple assay procedures for measuring the dietary content of the various tocopherols. The rôle of tocopherols as antioxidants in increasing the stability and utilization of carotene and vitamin A warrants extensive study.

Vitamin E and the Fat Content of Milk

Hickman and Harris¹⁸ and Harris *et al*¹⁹ have reported that feeding dairy cows one gram daily of natural mixed tocopherols as a supplement to the normal feed resulted in a marked increase in the fat percentage of the milk and, thus, an increase in the milk fat secreted. Attempts by Whiting *et al*,²⁰ Gullickson *et al*,²¹ and Phillips *et al*²² to confirm these results have been unsuccessful.

At the Cornell Station,²⁰ a study was carried out to determine whether an antagonistic action existed between tocopherols and cod-liver oil in milk fat synthesis. The theory seemed plausible, since it is established that feeding cod-liver oil causes muscle dystrophy in several species of herbivorous animals and that the trouble is preventable or curable by alpha-tocopherol. Furthermore, it is well known that feeding cod-liver oil to dairy cows will cause a marked decrease in the fat content of the milk.²³ If it were true that extra tocopherols would increase milk fat synthesis, it appeared just possible that these factors were acting antagonistically. A carefully controlled study failed to reveal any such relationship. The tocopherol supplements (TABLE 4) did not increase the fat content of the milk. Cod-

TABLE 4
INFLUENCE OF TOCOPHEROL AND COD-LIVER OIL SUPPLEMENTS ON THE FAT PERCENTAGE AND VITAMIN CONTENT OF MILK AND PLASMA

Supplement	Average milk		Tocopherols (μg./100 ml.)	
	Pounds	Fat per cent	Milk fat	Plasma
None	30.3	4.24	2990	582
Tocopherols	30.7	4.30	3569	735
Cod-liver oil	32.8	3.63	2529	427
Tocopherols and cod-liver oil	32.0	3.63	3590	696

liver oil decreased the fat percentage and tocopherols did not minimize this effect. Feeding tocopherols increased their content in the milk fat and the blood plasma of the cows, and feeding cod-liver oil appeared to decrease the tocopherol content of the plasma and milk fat.

Gullickson reported²⁷ that cows fed rations devoid of vitamin E appeared to produce milk of lower than normal fat content and that the addition of tocopherol supplements tended to increase the fat percentage of the milk. Thus, the question remaining finally to be answered is whether or not severe vitamin E deficiency caused a lowering of the fat content of milk. From the data now available, however, it appears doubtful that adding tocopherol supplements to average winter rations will increase the synthesis of milk fat by dairy cows, at least sufficiently to be of economic importance.

Vitamin E and Oxidized Flavors in Milk

In experiments at Cornell University, Krukovsky *et al*²⁴ showed that milk fat samples which were high in tocopherols were more resistant to the development of oxidized flavors. This aspect of tocopherol metabolism would appear to be of economic importance. Studies were therefore carried out to determine the normal tocopherol content of the milk fat of dairy cows as influenced by breed and by season and feed. Samples of milk were collected from the Cornell University herd during a yearly period, including two pasture seasons. The average data (TABLE 5) show that Guernsey

TABLE 5
THE TOCOPHEROL CONTENT OF MILK FAT

<i>Variable</i>	<i>Number of samples</i>	<i>Tocopherol content of milk fat</i>
		$\mu\text{g.}/100\text{ gm.}$
Breed:		
Holstein-Friesian	51	2219
Brown Swiss	33	2553
Guernsey	28	3033
Jersey	16	2624
Season:		
Winter	40	2087
Pasture	88	2736
Average	128	2533

cows secrete milk fat of higher tocopherol content than the other breeds studied and that Holstein-Friesian cows were lowest on the same feeds. During pasture feeding, all cows produced fat of appreciably higher tocopherol content than when they were fed hay and corn silage during the winter. These observations led us to study the tocopherol content and stability of milk produced by cows fed on different types of roughages. It was observed²⁵ that when cows were fed ladino clover hay their milk fat was lower in tocopherol content and it was more susceptible to the development of oxidized flavors than milk from cows fed alfalfa or timothy hay (TABLE 6). In further contrast, when birdsfoot trefoil (*Lotus corniculatus*) hay was

TABLE 6
THE TOCOPHEROL CONTENT AND STABILITY OF MILK FAT AS INFLUENCED BY VARIOUS TYPES OF HAY

<i>Type of hay</i>	<i>Tocopherol content of milk fat</i>	<i>Percentage of milk samples showing oxidized flavors</i>
	$\mu\text{g.}/\text{gm.}$	
Alfalfa	23.8	0
Timothy	22.0	0
Birdsfoot trefoil	28.3	0
Ladino clover	17.7	57

fed, the milk was unusually high in tocopherol content and exhibited superior stability. In repeating these studies, Krukovsky *et al*²⁶ have been able to confirm these observations and to show that specific types of pasture and hay crops influence the vitamin content and stability of milk. FIGURE 1 shows the distribution of milk samples according to stability and tocopherol content. Tocopherol appears to be one of the important nutrients deserving extensive consideration in any attempt to improve the quality and stability of milk.

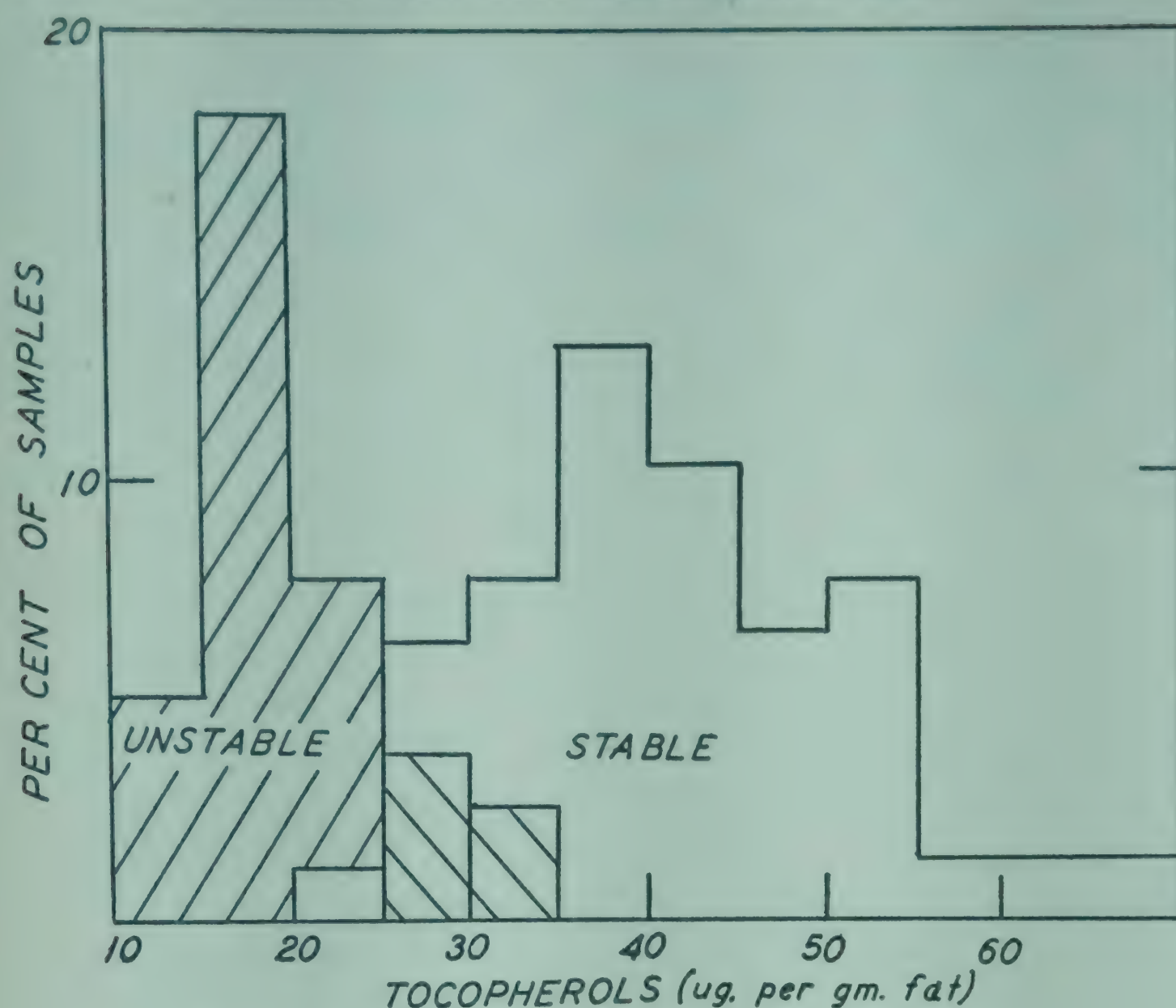


FIGURE 1. The distribution of samples of milk fat by tocopherol content.

Vitamin E Requirement

Not much is known about the quantitative tocopherol requirements of farm animals. In one study¹⁴ a daily intake of 2.3 mg. of total tocopherols per kilogram of body weight proved fully adequate for mature sheep. Two to 5.0 mg. of dl- α -tocopherol per kilogram of body weight daily will cure muscular dystrophy in lambs.² The minimum requirement for prevention of muscular dystrophy in lambs has been estimated¹⁴ to be between 0.23 and 0.37 mg. of tocopherols per kilogram of body weight daily. On the basis of analyses, it can be estimated that a daily intake of 4.0 mg. of tocopherol per kilogram of body weight as supplied by milk is well above the actual minimum requirement of dairy calves. For none of these animals has the minimum requirement been established, but the values shown above are not out of line with the known requirements of 0.3 to 1.0 mg. of tocopherols per kilogram of body weight daily for rabbits,^{29, 30} of 3.0 mg. for chickens,³¹ and 4-16 mg. per kilogram of body weight daily for ducks.³²

The recent evidence that tocopherols improve the stability of milk and other animal products²⁸ suggests that perhaps the tocopherol requirement of milk- and meat-producing animals should be judged on the basis of the amounts needed to impart desirable nutritional stability to the products used as food, rather than the smaller amounts which will prevent the onset of gross pathological changes.

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Discussion of the Paper

T. MOORE (*Dunn Nutritional Laboratory, Cambridge, England*): Does the tocopherol content of colostrum vary as widely as that of vitamin A and carotene?

J. LOOSLI: No, much less.

H. KAUNITZ (*Departments of Pathology and Animal Care, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): Was there evidence of virus disease involvement in the experimental lambs?

J. LOOSLI: No. But this merely raises the question regarding methods used to demonstrate viral infection.

K. HICKMAN: Corn and soybeans are largely replacing green alfalfa in animal feeds. What is the prospect that δ - and γ -tocopherols are displacing and competing with α -tocopherol?

P. HARRIS (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): Experiments with rats indicate that the non- α -tocopherols do not compete with α -tocopherol and cannot be considered as anti-vitamins.

W. GOVIER (*Department of Pharmacology and Endocrinology, The Upjohn Company, Kalamazoo, Mich.*): The non- α -tocopherols may decrease the absorption of simultaneously administered α -tocopherol, inducing the same results which would be obtained if the non- α -tocopherols were anti-vitamins.

P. HARRIS: Absorption or tolerance curves of humans given either α -tocopherol alone or α - plus relatively large amounts of non- α -tocopherols indicate that the absorption of α -tocopherol is not affected by the other tocopherols.

VITAMIN E IN THE NUTRITION OF FARM ANIMALS*

By D. B. Parrish

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Results of only a few investigations, conducted under controlled conditions, have indicated definite physiological needs for vitamin E by any of the common farm animals, cattle, sheep, goats, swine, and horses. Contrary to findings with laboratory animals, reproductive ability of these species has not been demonstrated to be dependent upon intake of vitamin E, but muscular disturbances and cardiac failure have been reported when rations were deficient in this vitamin. Since there is little information upon which to base statements of dietary requirements, consideration will be given primarily to physiological utilization of dietary tocopherols (vitamin E) by farm animals, especially by dairy cattle. Studies with poultry will not be considered at this time.

In order to study the effect of tocopherol supplementation on the concentrations of tocopherols in blood serum and in milk of dairy cows, a single-reversal trial,¹ consisting of two 9-week periods, was conducted using 14 cows which were paired by breed, lactation number, and previous production. All cows were fed a typical barn ration that included alfalfa hay, Atlas sorgo silage, and a grain mixture, and one cow of each pair received tocopherols at a daily rate of 1 g. per 1000 lbs. of body weight. Blood serum and milk tocopherol concentrations were determined at the start of the trial and at the end of each period. During periods of supplementation, tocopherol levels were increased approximately 40 per cent in serum and 50 per cent in milk. Changes for each group are shown in FIGURE 1. Feeding of tocopherol supplements as a method of enriching milk seems to be a wasteful procedure, since the increase represented a transfer to milk of less than one per cent of the supplement ingested daily. In other studies,² it has been observed that dry cows, grazing cereal-grass pasture, had about 50 per cent higher serum tocopherol levels than similar cows that had been restricted to barn rations for 10 days or more.

Studies of Harris *et al.*³ revealed a similar effect of tocopherol supplementation on concentrations of tocopherols in blood and in the fat from milk obtained in the winter. In summer, however, when cows received pasture, the milk-fat had approximately the same tocopherol content as that in the winter from supplemented cows, and supplements caused only small increases. Whiting *et al.*⁴ reported increased tocopherol contents in plasma and in milk from cows receiving 1 g. of supplement daily for 4 weeks. Kaay⁵ stated that cows on pasture had considerably higher serum tocopherol levels than those stall-fed and that administration of wheat-germ oil by mouth or injection caused no increases. He reported further that, in studies with mares, no relation of diet to levels of serum tocopherols could be found.

Changes in concentrations of serum tocopherols of cattle were found that seem to be related to stages of gestation and lactation (FIGURE 2).²

* Contribution No. 387, Department of Chemistry.

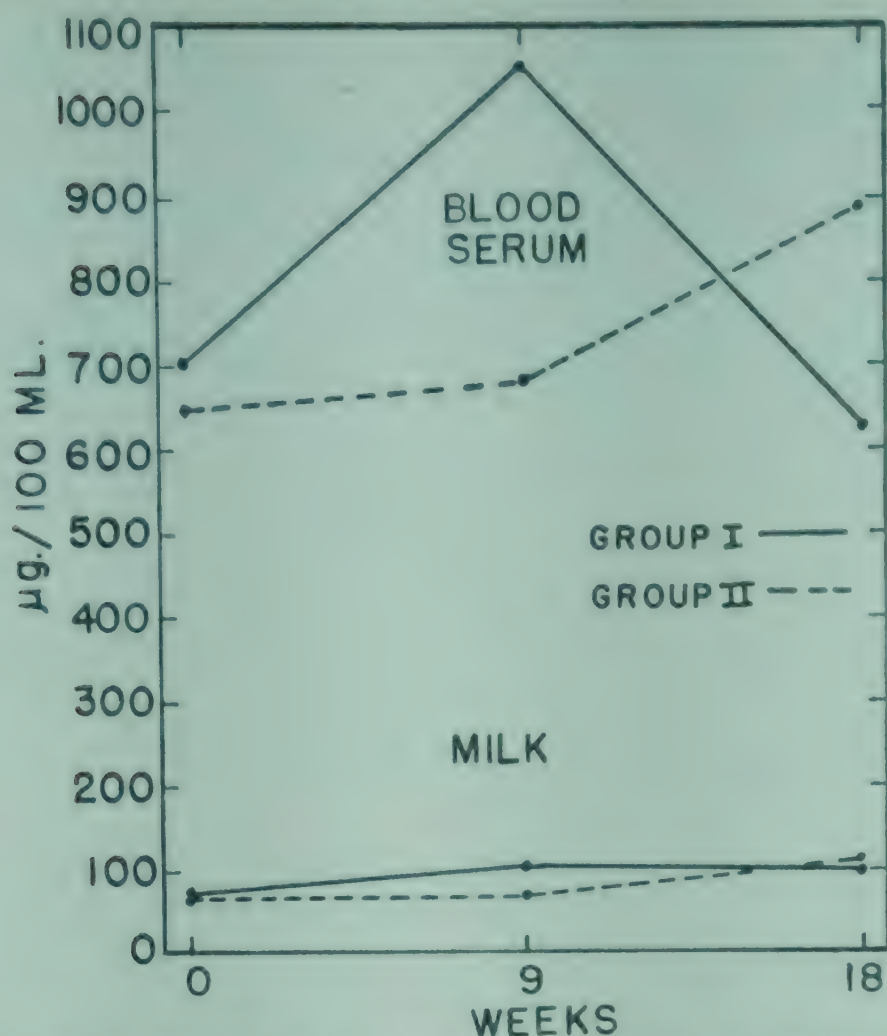


FIGURE 1. Tocopherol concentrations in blood serum and in milk of groups of cows receiving daily supplements of 1 g. of tocopherols per 1000 lbs. of body weight in a single-reversal trial. Group I received tocopherol supplement the first 9 weeks and Group II received it the last 9 weeks.¹

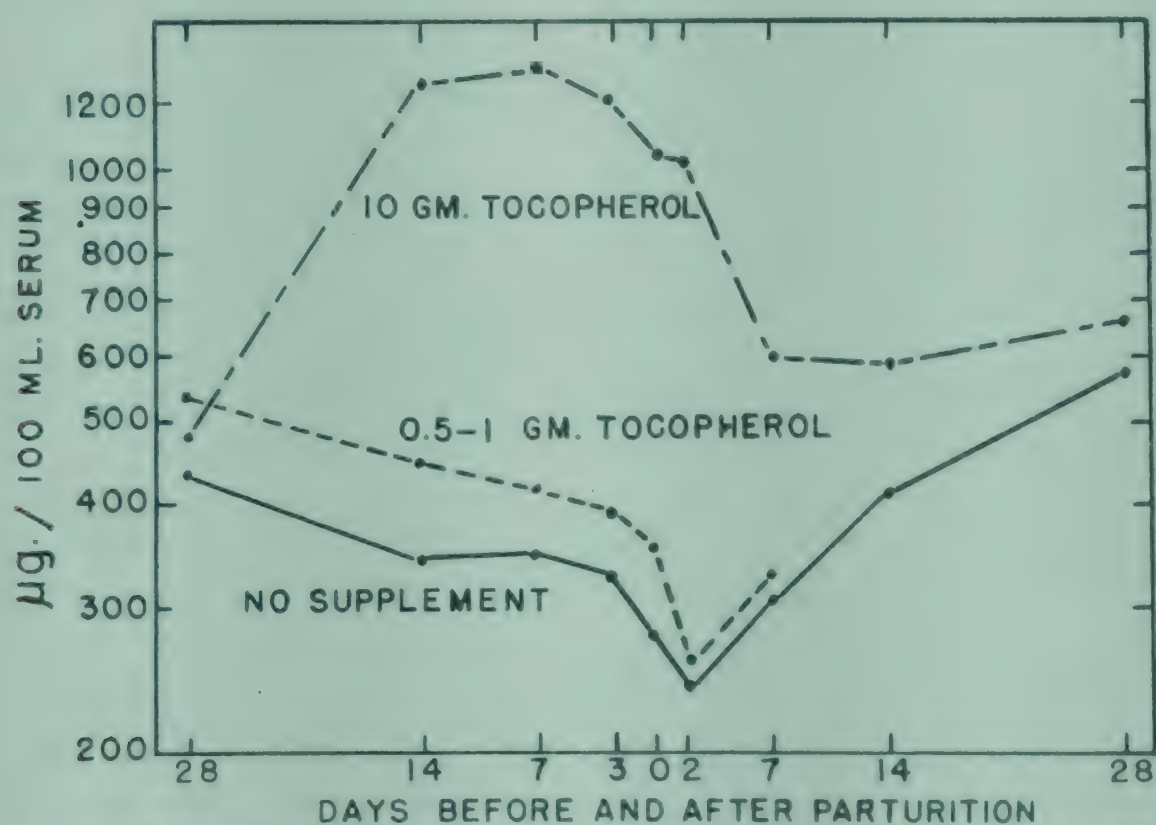


FIGURE 2. Tocopherol concentrations in serum from cows unsupplemented and supplemented with tocopherols during the terminal stages of gestation.² Levels of supplement given the cow are shown in the figure.

Levels in serum from cows in terminal stages of gestation that received unsupplemented rations decreased slowly until a few days before calving, at which time a more rapid change began. A minimum was observed on the second day *post partum*, followed by increases. Tocopherol levels were higher one month after calving than one month prepartal. Daily supplements of 0.5 g. of tocopherols from 28 to 14 days prepartal and 1 g. from

13 to 0 days increased concentrations of this vitamin in the serum somewhat but did not materially affect either prepartal or postpartal trends. However, feeding of 10 g. of tocopherols daily during the last month of pregnancy increased the levels in the serum over 400 per cent of that in the controls but did not prevent declines in the immediate parturient period. After 4 weeks of lactation, the cows supplemented at the higher levels had serum tocopherols only about 15 per cent higher than those not supplemented.

In investigations of placental and mammary transfer of tocopherols,⁶ it was found that calves whose dams received only barn rations were born with an average serum tocopherol level of 42 μ g. per 100 ml. After consumption of colostrum, content of this vitamin rapidly increased in the serum, levels about five times higher being reached by the third day (FIGURE 3). When the dams received prepartal supplements, calves were born

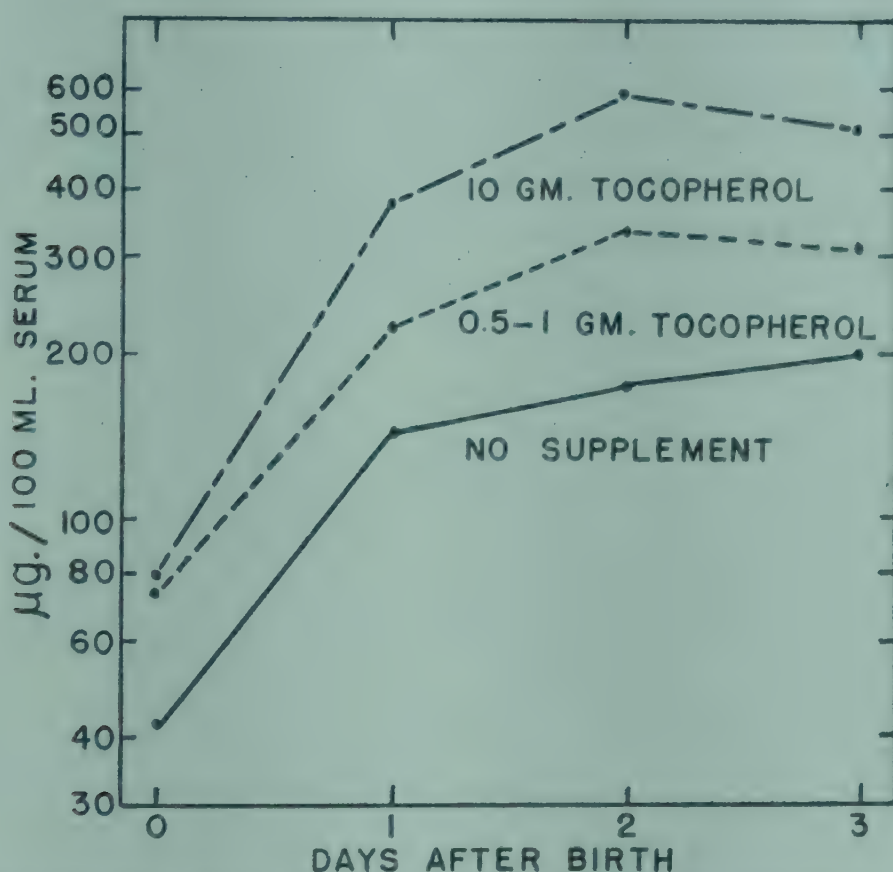


FIGURE 3. Serum tocopherol levels of calves receiving colostrum from dams unsupplemented and supplemented with tocopherols during terminal stages of gestation. Levels of supplements given the dam are shown in the figure.⁶

with serum tocopherols averaging almost twice as high as those of calves from unsupplemented cows. Giving cows 0.5 to 1 g. of supplement daily seemed to be about as effective as 10 g. daily in increasing serum tocopherol levels in calves at time of birth. Thus, serum tocopherol levels in calves were not proportional to those of the dams.

Calves receiving colostrum from supplemented cows had higher serum tocopherol levels than those from the controls (FIGURE 3). The highest values for the former calves were observed on the second day of life. Although within the first week after birth serum tocopherols of some calves rose to levels higher than those of their dams, the early accumulations did not have an appreciable effect on later serum concentrations of this substance. When calves received colostrum for three days, followed by whole

milk until the end of the second week, and then skim milk plus grain concentrate and hay *ad libitum*, serum tocopherol levels declined appreciably during the second to fourth weeks. Serum tocopherols of 3-week-old calves from supplemented and unsupplemented dams were nearly the same, levels of 198 $\mu\text{g.}$ per 100 ml. and 210 $\mu\text{g.}$ per 100 ml, respectively, being found.

Whenever calves were observed to have a severe diarrhetic condition, concentrations of serum tocopherols decreased. In one calf, a decrease of about 100 $\mu\text{g.}$ per 100 ml. of serum was observed from the first to the second day; in another case, a similar decrease was noted from the second to the third day. The cause of the decrease was not determined, but poor absorption due to the abnormal condition was suspected.

Since colostrum is a rich source of tocopherols, the rapid increases of this vitamin in serum of new-born calves is easy to explain. In a study of the concentrations of tocopherols in colostrum and early milk from unsupplemented and supplemented cows,⁷ it was found that, when only barn rations were fed, first colostrum was about four times higher in tocopherols than was milk from the same cows one week later. Prepartal supplements of 0.5 to 1 g. of tocopherols daily increased content of this vitamin in early colostrum about 40 per cent; 10 g. increased it fourfold. During the first four days of the transition from colostrum to normal milk, decreases in tocopherol levels in milk fat followed approximately a logarithmic curve. The rates of change were similar regardless of level of prepartal tocopherol intake of the cows.

It might be supposed that the transfer of tocopherols to the colostric secretions caused the decreases of serum tocopherols that were associated with stages of late gestation and early lactation which previously were mentioned. Apparently this will not account for all the change, since a similar trend was noted in levels of serum tocopherols of a pregnant mammectomized cow.⁸

Effects of supplementation on placental and mammary transfer of tocopherols also have been studied in sheep, goats, and swine.⁹ Giving the dams supplements of 80 mg. of tocopherols per 100 lbs. of body weight caused small, but nonsignificant, increases of tocopherols in livers of new-born lambs, kids, and pigs. Similar to observations on cattle, supplementation of the dams increased content of tocopherols in plasma of new-born lambs and kids (but not pigs) and in colostrum of sheep, goats, and swine.

From results of investigations summarized herein, it may be seen that tocopherol concentrations in serum of cows and calves are higher than those of any other fat-soluble vitamins,* the levels of tocopherols being about ten-fold higher than those usually found for vitamin A. It also is interesting that changes in tocopherols in the serum of pregnant and parturient cows, the relative importance of placental and mammary transfer of tocopherols to the new-born calf, and the effects of dietary supplementation with this vitamin are similar to the observations with respect to vitamin A. It therefore would be expected that tocopherols play an important part in the nutrition of farm animals. Nevertheless, perplexing problems arise when attempts

* The levels of the provitamin carotene are sometimes higher in certain breeds of cattle and might be considered an exception to this general statement.

are made to identify possible physiological functions of tocopherols in these animals.

No special reproductive difficulties have been noted in cattle¹⁰ and goats¹¹ fed vitamin E-deficient rations for several generations. Also pertinent is the report by Kaay⁵ that sterile cows and mares did not have particularly low serum tocopherols. At the present time the strongest indications that farm animals have definite need for tocopherols is found in the accumulating evidence that cattle and sheep are subject to disturbances of heart and skeletal muscle when rations contain insufficient vitamin E.^{12, 13}

Investigations of the sparing action of tocopherols on vitamin A and of the improvement in health and performance by use of tocopherol supplements generally have led to negative results. No substantial increases in vitamin A of blood serum, colostrum, and milk of cattle, sheep, goats, or swine have been observed when tocopherols were fed.^{1, 3, 4, 14, 15} Only in sheep has placental transfer of vitamin A been reported to be increased by use of tocopherols.¹⁴ Recent reports^{1, 4, 15, 16} do not confirm the finding of increased output of fat or "4 per cent milk"³ following use of tocopherol supplements. Growth and general health of calves were not improved by adding supplemental tocopherols to a typical ration fed calves from the fourth to the sixty-fourth day after birth.¹⁷

Thus, there is much evidence indicating that laboratory animals and farm animals do not respond similarly to dietary tocopherols. The situation is not unique for this vitamin, since nutrition investigators previously have been confronted with it with respect to proteins and the B-complex. In most cases, satisfactory explanations for these differences later emerged. Clarification of the rôle of tocopherols might result from further studies of differences in response of ruminants and non-ruminants to vitamin-E deficient rations. It seems unlikely, however, that rumen synthesis of vitamin E plays an important rôle, since tissues of animals apparently healthy on an E-deficient regimen were so low in this vitamin they would not support reproduction of rats.^{10, 11}

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THE RELATION OF VITAMIN E TO REPRODUCTION IN DAIRY CATTLE

By Thor W. Gullickson

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Breeding troubles are a constant source of worry and economic loss to dairy farmers and cattle breeders in general. The experiment which I am going to discuss briefly was started over ten years ago, largely as a result of a popular demand by dairy farmers for definite information concerning the relation of vitamin E to reproduction in dairy cattle. Sporadic attempts had been made to obtain information on this question by adding some vitamin E-rich supplement to normal rations, but results were largely inconclusive.

In this experiment, the plan was to start with young calves and to feed them throughout their entire lives on rations as nearly as possible vitamin E-free but providing enough of all other known essential nutritional factors.

Fifteen calves of mixed breeding, nine females and six males, were included in the original group. Later, several more calves of both sexes were added, along with the descendants of animals in the original group. A total of 36 animals was used in the experiment for various periods of time. This included four positive controls, consisting of 1 bull and 3 heifers, which were fed exactly like the others, except that each of them received a supplement of approximately 5 mg. tocopherol per kg. of body weight per day.

Ration Fed. Considerable difficulty was encountered in finding suitable feedstuffs that are vitamin E-free. It was found that all feedstuffs commonly fed to cattle are relatively rich in this vitamin. A modification of the Palmer bioassay method was followed in testing foodstuffs for their vitamin E content. By this method, sexually mature female rats that had been fed normal rations throughout the entire period of growth and their first gestation were placed on the basal vitamin E-free ration immediately after the birth of their litters. After weaning at 21 to 25 days, the young were reared to sexual maturity on the basal vitamin E-free ration, modified so as to contain such percentage of the foodstuff tested as was likely to be present, on the dry matter basis, when fed in maximum amounts to cattle on experiment. Each foodstuff was tested in this manner, and some were tested again in combination with others to determine their additive effects, if any, on reproduction. If the product was highly indigestible for rats, a benzene extract of it was made, and this was then incorporated at high levels in the basal vitamin E-free ration which was fed during the gestation period of the vitamin E-deficient rats. The rats so produced were mated when about 90 days old and thus received the test feed for periods of 9 to 10 weeks. Wheat germ oil of known potency was employed for the positive controls. A total of 3.5 to 4.0 milliliters was incorporated in the basal ration over a period of 6 to 7 weeks, corresponding to the minimum period the test feeds were consumed. The animals were sacrificed on the 21st day of pregnancy and the living and dead young and resorptions were

counted *in utero*. A less complete study was made, with male rats, of the ability of some of the products to prevent the characteristic testicular degeneration. After the ingredients in the ration had been selected, all new lots obtained were tested and found vitamin E-free before they were fed.

The ration fed consisted of rice straw as the sole roughage and a concentrate mixture made up approximately as follows: 25 per cent polished rice, 30 per cent brewers' dried grains, 18 per cent distillers' grains (solvent extracted), 11 per cent corn starch, 9 per cent dry skin milk, 4 per cent lard, 2 per cent steamed bone meal, and 1 per cent iodized salt. Delsterol (2000 D) was added as a source of vitamin D. A vitamin A concentrate was fed once daily to each animal at the rate of approximately 10,000 I. U. per 100 lb. of weight.

All calves were fed whole milk until about 3 weeks old, followed by skim milk to about 6 months. Rice straw was fed *ad libitum*, along with enough concentrates to provide the protein and energy required according to the Morrison standard.

Tests, made on feces from cattle fed vitamin E-poor rations as well as on the feces from similar animals fed normal rations, indicated that vitamin E is not synthesized within the digestive tract of ruminants.

Cattle were kept isolated from others. They were turned out for exercise in a vegetation-free lot almost daily. Shavings and waste rice straw was used for bedding.

Growth. All animals were weighed at birth or when placed on experiment and subsequently at 30-day intervals. The records show that cattle grew at a normal rate or above.

Reproduction and Breeding Ability. The cattle were observed daily for those manifestations which indicate development and functioning of the organs of reproduction. Sexual development and behavior in the bulls was tested by permitting them to mingle with females showing estrus. Beginning at about 6 months of age, all bull calves receiving the E-free ration invariably showed marked libido on such occasions. Studies made of semen samples showed that all ejaculates were normal in sperm activity, morphology, and longevity.

In females, studies of sexual development included observations for both physical and psychological signs of estrus, as well as rectal examinations of the uterus and ovaries for evidence of ovulation. These showed that the estrus cycle, with all its characteristic and continuous changes, including ovulation, occurred regularly and in a normal manner, starting when heifers were 7 to 9 months old.

The breeding records show that the reproducing ability of the cattle fed vitamin E-poor rations was not adversely affected through three generations. A total of only 30 services produced 25 pregnancies in the 19 females of breeding age that were fed E-poor rations, or an average of only 1.2 service per conception. All F_1 and F_2 heifers so fed conceived on the first service and all heifers dropped their first calf at about 2 years. One F_1 heifer was only 17 months old and another was only 16 months old when they calved. An F_1 bull calf was used successfully when only about 10 months old, thus indicat-

ing that feeding of vitamin E-poor rations did not delay sexual maturity. Furthermore, one cow gave birth to 3 normal calves within a period of 25 months, with only 10 months between the last two parturitions. There were no abortions and all gestations were normal in length, averaging 280 days. All calves born appeared to be normal in vigor at birth, and fetal membranes invariably were expelled within several hours after calving occurred. The veterinarians reported that no abnormalities were found in the reproductive organs of any of the animals that died or were slaughtered.

Physical Condition. The cattle fed the vitamin E-poor ration displayed few if any abnormalities in action or appearance. Thirteen out of the 28 animals so fed for one year or more died suddenly at ages ranging from 21 months to 5 years. One of these was a bull about 30 months old. These animals displayed few or no premonitory symptoms of their impending death. Several collapsed while consuming their rations. A few of them showed slight loss of appetite during the month or more before their death. One F₂ heifer showed profuse salivation with complete anorexia several weeks before she died. Several days before her death she became too weak to stand. This heifer also differed from others in that at birth her hocks were slightly swollen, a condition that persisted. The only bull which died suddenly showed some loss of appetite. He lost about 70 pounds in weight during the last four months of his life.

In no case did gross post-mortem examinations reveal pathological changes sufficiently severe to indicate the specific cause of death. Slight hemorrhages were found in the brains of some of the cattle and, in others, they were apparent on the bowels and occasionally on the heart and pancreas. As we reported previously, electrocardiograms obtained on some of the animals during several months before their death revealed that a gradual and progressive change occurred in cardiac functioning, suggesting heart failure as the most probable cause of death.

Milk and Fat Production. Mild and butterfat records were kept on each animal. Feeding of the vitamin E-poor ration did not appear to affect the volume of milk produced but seemed to have a depressing affect on its fat content.

Conclusion

In conclusion, it should be pointed out that, although the feeding of vitamin E-poor rations did not appear to affect the ability of cattle to reproduce, it is significant that out of the dozen or more animals which died suddenly six had been pregnant 6 to 8 months at time of death and three died within 3 days after calving. However, pregnancy and parturition probably were only indirectly involved in causing these deaths. A critical vitamin E shortage would be expected to develop during the last several months of the gestation period, when the requirement for one or more essential nutritive factors has been shown to increase greatly. In rats, during the corresponding period, degenerative changes occur, not in the deficient mother but in the developing embryo which eventually succumbs and is absorbed. Mason has shown that placental transmission of vitamin E in the rat is very

small. Is it possible that in the bovine it is greater, resulting in the mother being sacrificed instead of the developing fetus? Such a species difference is suggested by the recent work of Whiting and Loosli in experiments with sheep, goats, and swine. It has also been shown that deficiencies of protein and various minerals affect the cow more seriously than it does her unborn calf.

The increased burden imposed during the stress of calving and initiation of lactation are other factors that would be expected to affect the already injured heart and the welfare of the cow during this period. Brody has shown that gestation increases heat production in cattle about 40 per cent above the non-gestating level during the last one-third of the gestation period and, also, that heavily lactating cows produce about twice as much heat under normal feeding conditions as when not milking. Pulse rate, respiration rate, and ventilation rate were found to parallel the course of heat production.

Discussion of the Paper

DOCTOR EVAN SHUTE (*The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): It is well known among obstetricians that pregnancy is the test par excellence of cardiac function, so it was with great interest that we learned that Dr. Gullickson's cows died of heart failure, especially when pregnant.

Did any of the cows receiving tocopherol supplements die of cardiac failure? What were the microscopic findings in the heart on autopsy?

DOCTOR GULLICKSON: None of the control animals died. There were no further histopathological results than were reported in the preliminary report in *Science* **104**: 312. 1946.

DOCTOR Z. MENSCHIK: What changes, if any, were noted in the embryos or fetuses of the vitamin E-deficient cows?

DOCTOR GULLICKSON: No changes were observed.

DOCTOR P. L. HARRIS (*Research Laboratories, Distillation Products, Inc. Rochester, N. Y.*): In agreement with our earlier observations, it is interesting to note that milk fat production is probably related to vitamin E intake when the level of tocopherol ingestion is below the optimum.

RESPONSE OF SWINE TO VITAMIN E-DEFICIENT RATIONS*

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An early study of vitamin E deficiency in the chick embryo¹ showed that death of the embryo was usually accompanied by hemorrhage and that the site of bleeding presented a characteristic histological picture (FIGURE 7). A similar condition was later shown to occur in supposedly normal chick embryos.² Since then, Mason³ has shown that hemorrhage frequently occurs in vitamin E-deficient rat fetuses, although no histologic reaction was reported at the site of hemorrhage. It has also been found by Adamstone that hemorrhage may occur in pig embryos, the condition having been first observed in routine laboratory class study of 10 mm. pig embryos (FIGURE 6). In this case, moreover, the histological picture is almost identical to that seen in the chick (cf. FIGURES 6 and 7), and, frequently, clusters of degenerating pycnotic cells are found in blood vessels throughout the body (FIGURE 8), just as also occurs in the chick. It was recognized, of course, that the condition observed in the pig might not be related to vitamin E deficiency, but examination of about 600 embryos revealed hemorrhage in approximately 10 per cent of the cases. Hence, hemorrhage is undoubtedly an important cause of fetal death. Therefore, an investigation of the possibility that vitamin E deficiency is involved seems justified.

Plan of the Experiment

(a) *Animals and Management.* A group of nine gilts (7 Duroc Jersey and 2 Poland China) were selected for the test and were divided into 3 lots. These gilts averaged 70 pounds in weight at the beginning of the experiment and had been raised in continuous confinement on concrete floors. For 75 days prior to the beginning of the experiment they had been fed a well-balanced ration. During the entire vitamin E test, the gilts were also confined on concrete floors and were hand-fed twice daily.

(b) *Experimental Rations.* Since large quantities of food are necessary for such an experiment, a natural ration was used rather than a more costly, purified diet. The ingredients, composition, and vitamin content of the basal ration used in the experiment are given in TABLE 1. This was fed to the gilts in Lot 1. The ration contained more than the nutrient allowances recommended by the National Research Council,‡ and all rations used were adequate except for vitamin E. The tocopherol content of this ration was of the order of 1.4 mg. per pound of food. Thus, since an average of 5.7 pounds of food was consumed by each pig per day, the total daily intake of vitamin E was quite low.

In Lot 2, the basal ration was supplemented with 10 per cent rancid lard to ensure further depletion of vitamin E. In Lot 3, the basal ration

* This investigation was supported by funds contributed by the Viobin Corporation, Monticello, Illinois and by Hoffman-La Roche, Inc., Nutley, New Jersey, who also donated the α -tocopheryl acetate used in the experiment.

† With the technical assistance of C. A. Blomquist

‡ National Research Council Bull. II. Recommended Nutrient Allowances for Swine. 1944.

TABLE 1
BASAL-RATION FED

Ingredients	Pounds	Composition and vitamin content	
Clear wheat flour	86.0	Protein, %	21.7
Crude casein	4.0	Fiber, %	.6
Yeast, dried brewer's*	8.0	Calcium, %	.63
Minerals (H & K)†	2.0	Phosphorus, %	.63
Total	100.0‡	Manganese, p.p.m.	34.0
		Thiamin, mg. per lb.	8.68
		Riboflavin, mg. per lb.	2.54
		Niacin, mg. per lb.	3.89
		Pantothenic acid, mg. per lb.	10.53
		Total tocopherols, mg. per lb.	1.39§

* "Strain S," Anheuser-Busch, Inc.

† Composition of H & K Mineral Mixture (% or lbs.):

Iodized salt	25.0	Potassium carbonate	0.2
Steamed bone meal	29.0	Copper sulfate	0.1
Ground limestone	40.0	Manganese sulfate	0.56
Magnesium carbonate	4.0	Cobaltous chloride	0.10
Ferrous sulfate	1.0	Zinc carbonate	0.04

‡ Fortified marine liver oil (vitamin E-free) was fed as 1 per cent of the daily feed three times weekly to all lots (Mon., Wed., Fri.). It contained 3,000 I.U. vitamin A and 300 U.S.P. units of vitamin D per gram of oil. Made by Distillation Products, Inc., Rochester, New York.

§ Assayed by the ferric chloride-a, a' dipyridyl method, courtesy of Dr. J. C. Bauernfeind, Hoffman-La Roche, Inc., Nutley, N. J.

was supplemented with dl- α -tocopheryl acetate so as to supply 50 mg. per head daily, the supplement being fed on Tuesday and Thursday. All gilts grew well and thrived on the experimental rations, as shown in TABLE 2.

TABLE 2
RESULTS DURING GROWTH

Lot number	1	2	3
Treatment	Basal	Basal 90%; rancid lard 10%	Basal + dl-to- copherol acetate*
Number of gilts	3	3	3
Av. initial wt., lbs.	70	73	70
Av. final wt., lbs.	256	277	250
Av. daily gain, lbs.	1.68	1.83	1.62
Av. daily feed per pig, lbs.	5.74	5.63	5.78
Feed eaten per 100 lbs. gain, lbs.	341	308	357

* Fed to supply 50 mg. of alpha-tocopherol per head daily, although it was fed twice weekly (Tuesday and Thursday).

After farrowing, the sows' udders were swabbed once daily during lactation with a saturated solution of copperas (1 pound in 3 pints of water) to prevent nutritional anemia in the pigs.

(c) *Matings.* At the age of about 8 months, the gilts were bred to fertile boars from the Illinois Station herds. All gilts were allowed to farrow and go through one lactation. When their pigs were weaned at the age of 8 weeks, the sows were re-bred. After each sow had passed through the next expected estrus period without showing signs of heat, thus indicating that pregnancy had occurred, she was slaughtered, and the reproductive tract

(including embryos, if any) and samples of other tissues were removed for histological study. It was hoped by this means to obtain embryos which might show a hemorrhagic condition similar to that observed in laboratory material. When the pigs of the first litters were weaned, some of them were also sacrificed so that tissues could be obtained for histological study.

Experimental Results

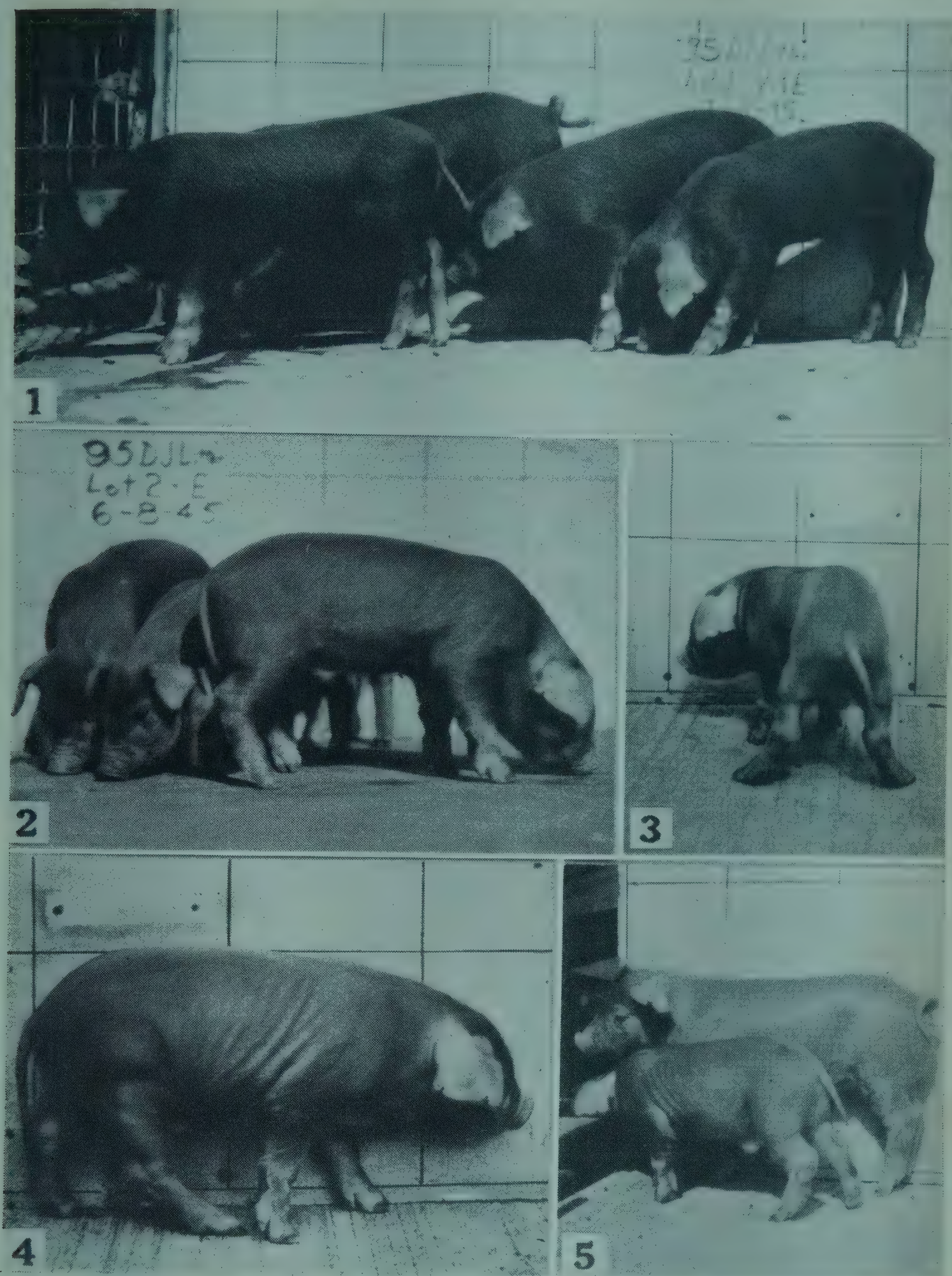
Gestation and Lactation Records. The gestation and lactation performance of the three groups of sows and their litters are summarized in TABLE 3. Comparison of the three lots is difficult because of the fact that only one

TABLE 3
GESTATION AND LACTATION RESULTS

<i>Lot number</i>	1	2	3
<i>Treatment</i>	<i>Basal</i>	<i>Basal 90%;; 10% rancid lard</i>	<i>Basal + dl-to- copheryl acetate</i>
<i>Gestation Results:</i>			
No. of gilts started	3	3	3
No. of gilts that farrowed	1	2	3
Av. initial wt. per gilt, lbs.	267	335	285.6
Av. final wt. per gilt, lbs.	495	524	484
Av. age in days at farrowing	347	367	367
Av. daily gain to farrow, lbs.	2.19	1.78	1.67
Av. daily feed consumed			
Basal, lbs.	6.46	—	7.10
Basal + rancid lard, lbs.	—	6.39	—
Marine liver oil, lbs.	.030	.030	.030
dl, α -tocopheryl acetate, cc.	—	—	1.92
Av. no. pigs farrowed per litter	9.0	6.5	8.3
Av. birth wt. per pig, lbs.	2.41	2.94	2.88
Percent of pigs farrowed			
Strong	78	92	88
Medium	22	0	4
Weak	0	8	4
Dead	0	0	4
Immature	0	0	0
<i>Lactation Results:</i>			
Av. no. pigs weaned per sow farrowed	9	2	4.7
Av. 21-day weight, per pig, lbs.	10	11.25	11.25
Av. 56-day weight, per pig, lbs.	20.2	19.25	24.04
Av. daily ration, sow and litter			
Basal, lbs.	7.0	—	7.62
Basal + rancid lard, lbs.	—	4.3	—
Marine liver oil, lbs.	.031	.030	.033
dl, α -tocopheryl acetate, cc.	—	—	2.04

sow in Lot 1 farrowed and because one sow died in Lots 1 and 2 during the first pregnancy.

Lot 1: All pigs farrowed by the single sow in this lot were successfully weaned. Nevertheless, at 27 days of age, 2 of the 9 pigs showed definite lack of muscular control in the hind legs. Growth rates were poor and, at 52 days of age, all 9 pigs showed wobbly gaits, incoordination of the hind legs,



FIGURES 1-5

FIGURE 1. Litter of Duroc Jersey pigs in Lot 1. Note rough hair coats.

FIGURES 2, 3, 4. Pigs from litters of Lot 2. Note rough hair coats and weakness of hind legs, particularly evident in FIGURE 3.

FIGURE 5. Contrast between normal pig of Lot 3 and pig of same age from Lot 2. Normal pig considerably larger and more alert than E-deficient pig.

and rough hair coats (FIGURE 1). They also fatigued easily when forced to move about the pen. Finally, 4 pigs (2 males and 2 females) were sacrificed and tissue samples obtained for histological study.

Lot 2: In this lot, the Poland China gilt (Sow #1) farrowed 6 live pigs, but, within 48 hours, all were dead. The sow had difficulty in rising and appeared to lack muscle tonus. Since this sow did not improve, she was slaughtered 29 days after farrowing. The Duroc Jersey (Sow #2) farrowed 7 strong pigs, but, at three days, all showed rough hair coats and appeared unsatisfied after nursing. After 5 days, 1 pig showed incoordination and, at the end of the first week, only 4 survived. At 9 days, all of these pigs showed wobbly gaits and spraddled while nursing. They walked with a semi-crouch of the hind legs and failed to play. They had weak backs, lacking the normal arch. The appearance of these pigs is shown in FIGURES 2-4.

Lot 3: All pigs raised by gilts in Lot 3 (14 out of 25) were normal when weaned. They were heavier, thriftier, and more sleek than the pigs in Lots 1 and 2 and, in addition, showed no evidence of muscular incoordination (the larger pig in FIGURE 5).

It is evident from the data presented above that the records of farrowing and lactation of the pigs in Lot 3 were definitely better than those of Lots 1 and 2. This indicates that a ration low in vitamin E has a harmful effect on the reproductive performance of the pig and, furthermore, that it produces definite injurious effects, particularly muscular weakness.

Vitamin A Stores in Livers of Sows

Liver samples from two sows in each lot were assayed for their content of vitamin A. The values obtained, in I.U. of vitamin A per grain of fresh liver, were as follows: Lot 1, 1400 I.U.; Lot 2, 1109 I.U.; and Lot 3, 1275 I.U. These values indicate adequate liver storage of vitamin A. Hence, the results of the experiment were apparently not complicated by vitamin A deficiency.

Histological Observations. Following the weaning of the young pigs from the first litters, the sows were re-bred and were slaughtered after they had passed one heat period without showing signs of estrus. The tissue samples secured from the sows and pigs were fixed in Bouin's solution and, after sectioning, were stained with Harris's haematoxylin and eosin or with Heidenhain's haematoxylin. The histological findings in the reproductive organs of the adult females, the testes of the young males, and also the liver and muscle are of particular interest and will be described. Other organs, including kidney, pancreas, spleen, heart, lung, spinal cord, sciatic nerve, and bone marrow, showed no evident abnormalities.

FIGURES 6-13 (*See opposite page*)

FIGURE 6. Hemorrhagic site in posterior vena cava of 10 mm. pig embryo. Note rupture of blood vessel and accumulation of pycnotic cells. (See arrow.) $\times 95$.

FIGURE 7. Hemorrhagic site in wall of atrium of heart in 72 hr. chick embryo. Note accumulations of pycnotic cells. $\times 950$.

FIGURE 8. Peculiar cluster of degenerating pycnotic cells in 4th aortic arch of 10 mm. pig embryo. Similar clusters were found in blood vessels in all parts of the body. $\times 950$.

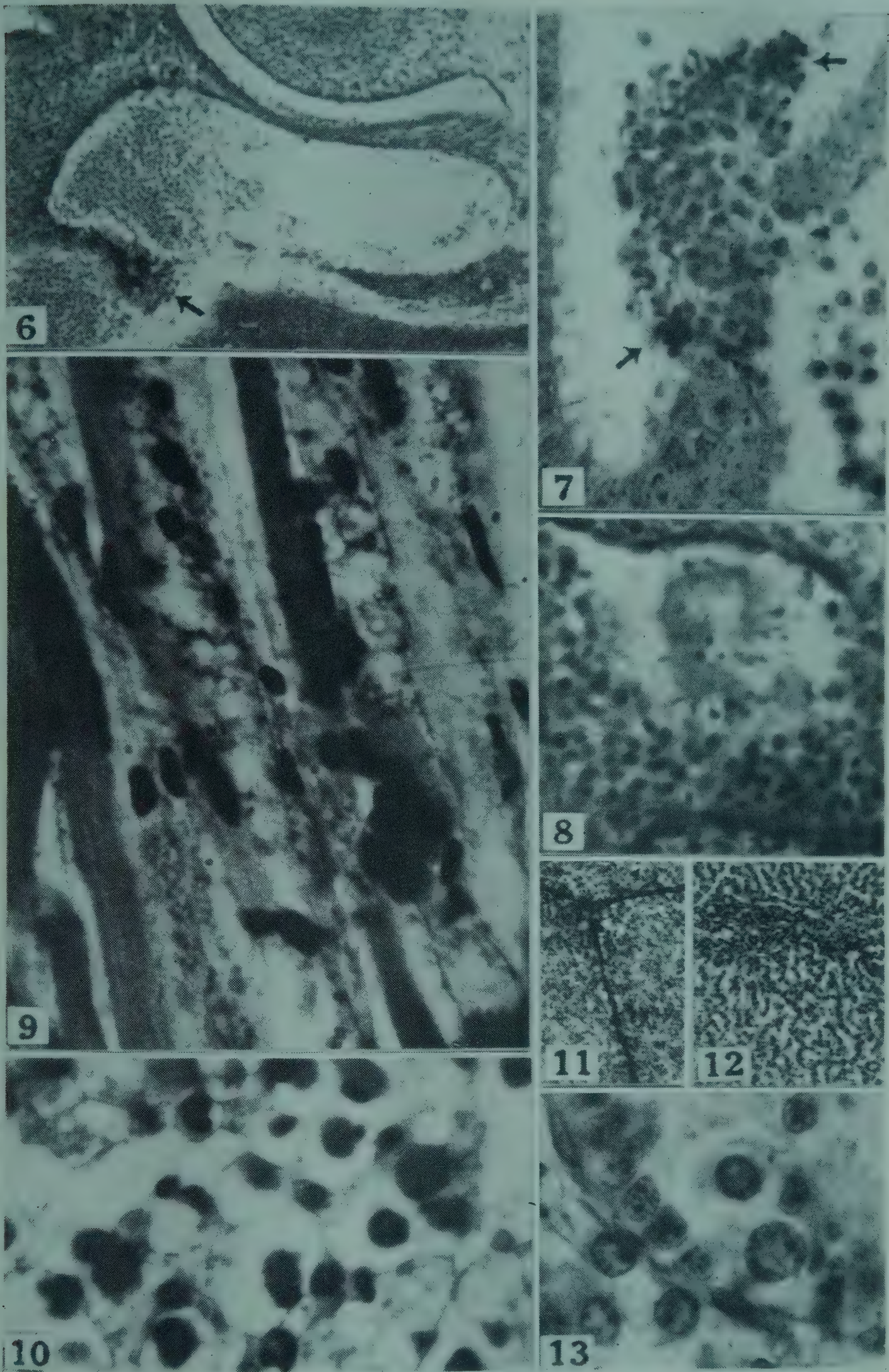
FIGURE 9. Degenerating muscle fibers in muscle from pig of Lot 2 (longitudinal section). $\times 950$.

FIGURE 10. Degenerating muscle fibers in x section. Shrinkage of some fibers and complete disorganization of others can be seen. $\times 950$.

FIGURE 11. Interlobular connective tissue septum in normal liver. $\times 95$.

FIGURE 12. Thickened septum in liver of pig from Lot 2. $\times 95$.

FIGURE 13. Small portion of seminiferous tubule showing one normal primary spermatocyte and another which exhibits shrinkage of cytoplasm and pynotic condition of the nucleus. $\times 950$.



FIGURES 6-13 (For description see facing page)

(a) *Reproductive Organs of Females.* Lot 1: Sow #1 died during the first pregnancy from some undetermined cause. The ovaries were in an advanced stage of degeneration amounting almost to castration. Seven corpora lutea were present in the left ovary and 2 in the right, but these showed extensive infiltration of connective tissue and disintegration of luteal cells. All corpora were soft and mushy. The stratum granulosa of the follicles was also disintegrated, and primordial follicles were almost completely absent.

The uteri of this animal contained a total of 8 blastocysts, all of which were in process of degeneration. Embryos were recovered in 5 of these, but they were in different stages of development. Disintegration was particularly marked in the brain and neural tube. No traces of embryos were found in the remaining 3 blastocysts.

Sow #2, after the second mating, contained 9 normal embryos approximately 12 mm. in length. The ovaries of this female were normal.

In sow #3, no embryos were found, but the ovaries contained large corpora lutea which were evidently degenerating and contained conspicuous blood clots.

The parts of the reproductive tract, namely, uteri and oviducts, of all three sows in this group were apparently normal.

Lot 2: Sows 1 and 2, which had farrowed 6 and 7 pigs respectively at the first mating, failed to become pregnant at the second and later matings. On post-mortem examination, the uteri and oviducts of both animals were normal and the ovaries contained regressing corpora lutea. Sow #3 died 32 days after the first mating but no embryos were recovered, although the ovaries contained regressing corpora lutea.

Lot 3: In this lot, two of the sows which had farrowed 25 pigs were pregnant after the second mating and contained 14 and 15 normal embryos. Histologically, the uteri, oviducts, and ovaries were normal. The third sow failed to become pregnant, but post-mortem examination showed that both ovaries contained large cystic follicles.

Although reproductive failure occurred in the vitamin E-deficient sows, examination of the one lot of embryos failed to disclose any evidence of the hemorrhagic condition which was anticipated (Sow 2, Lot 2).

(b) *Liver.* As was to be expected, the liver tissues of the sows from Lot 3 were normal and contained moderate amounts of fat. By contrast, the livers of the sows from Lots 1 and 2 showed irregular distribution of fat concentrated in more or less localized areas. In addition, some, though not all, of these liver tissues showed extremely broad connective tissue septa between the lobules (cf. FIGURES 11 and 12). The significance of this condition is not apparent. It was also found that the Poland China sow of Lot 2 had pools of blood accumulated in many of the liver lobules, accompanied by degeneration of the hepatic cords.

The livers of the young pigs from Lots 1 and 2 showed considerable accumulation of fat but were normal in other respects.

(c) *Muscular Tissue.* Most of the young pigs of Lots 1 and 2 showed leg weakness and muscular incoordination. There was, however, considerable variation as to the degree of muscular involvement, ranging from: (1) stages showing merely an increase in numbers of nuclei, and (2) shrinkage of individual fibers, to (3) extensive necrosis and degeneration of muscle fibers in the most severe cases (FIGURES 9, 10). In these, the fibrils were fragmented into small granules irregularly dispersed throughout the sarcoplasm; and, in some cases, the fibrils had completely disappeared. By contrast, muscular tissues from the pigs of Lot 3 were normal.

(d) *Histology of the Testis.* Testicular tissue was available from 4 of the young pigs of Lots 1 and 2 and from 8 others which were castrated at the age of 8 weeks. In all cases the tubules were very immature, but in many instances primary spermatocytes had developed, although the lumen of the tubules were full of a coagulum. Many of these primary spermatocytes showed shrinkage of the cytoplasm accompanied by the development of an acidophile condition which was indicated by a marked affinity for eosin. The nuclei of these cells were also considerably shrunken, homogeneous in appearance, and heavily stained with haematoxylin, thus showing the onset of a typical condition of pycnosis.

Discussion. The reproductive record of the sows in Lots 1 and 2 was very poor by comparison with the sows in Lot 3. Only one litter was secured in Lot 1, and, in Lot 2, only 4 pigs of the 13 farrowed survived until weaning. Nearly all of these pigs showed muscular weakness. Histological study of the reproductive organs of the sows in Lots 1 and 2 indicates

that these organs were apparently uninjured. Moreover, the condition of the ovaries suggests that there has been no interference with ovulation, which is typical of the reaction of the female mammal to vitamin E deficiency. It is not improbable, therefore, as indicated by the findings in the sows of Lots 1 and 2, which died early in the first pregnancy, that the embryos of the second pregnancy in the surviving sows had probably undergone resorption.

As for the conditions encountered in the liver and testis, it is undesirable to ascribe too much significance to them, although the condition in the testis suggests the onset of degenerative changes. As for the occurrence of heavy, interlobular connective tissue septa in the liver, its significance is not understood. A similar condition has been described by Morrione⁴ accompanying experimental cirrhosis induced in the rabbit by carbon tetrachloride.

The occurrence of muscular degeneration is a reaction of great importance. This reaction has been found in a considerable variety of experimental animals, as shown in the extensive review by Pappenheimer.⁵ The occurrence of so-called stiff-lamb disease appears to be another manifestation of a muscular disorder attributable to vitamin E deficiency.⁶ In swine, Ensminger⁷ described muscular disorders associated with deficiency of B vitamins, and Bueno⁸ reported a paralytic condition which he attributed to lack of some unknown factor in green food. Mason⁹ has also described necrosis in the skeletal muscle of the monkey which appears to resemble that encountered in the pigs in the present experiment.

Summary and Conclusions

The experiment reported in this article shows that, as a result of feeding swine on a vitamin E-deficient diet, the following effects are produced:

(1) The reproductive performance of sows is greatly lowered, apparently as a result of death of embryos rather than through any interference with ovulation and implantation, just as has been found in the E-deficient rat. No evidence was obtained as to the relation of hemorrhage to death in E-deficient embryos.

(2) Pigs from sows which were reared on the deficient diet exhibit muscular incoordination caused by disintegration and necrosis of the muscle fibers.

(3) Abnormalities observed in the liver are not regarded as of diagnostic significance; and the effects on the testis were not extensive, because of the immaturity of the animals.

(4) Vitamin E appears to be necessary to maintain normal health and growth in the young pig, although the results of the present experiments are not regarded as absolutely conclusive.

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EFFECT OF TOCOPHEROLS ON VITALITY OF PIGS IN RELATION TO "BABY PIG DISEASE"*

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The rôle of tocopherols in the nutrition of swine has not been adequately investigated. When it became apparent that vitamin E was essential for normal reproduction of small animals, investigations were carried out with large animals to determine whether supplementary tocopherols were required under practical conditions. Experiments with swine indicated that, under field conditions, tocopherols were not a limiting dietary factor. Because of these findings and the fact that tocopherols are present in all cereal grains and grasses, little emphasis has been placed on the rôle of this vitamin in swine nutrition.

The recent finding that supplemental tocopherols increase the fat content of cow's milk¹ indicated that the tocopherols might also be important in the nutrition of swine. If tocopherols would have a similar effect on lactating swine, the growth and well-being of pigs during the nursing period might be enhanced, due to the increased fat content of the dam's milk. The experiments to be described here were conducted to determine the effect of dietary supplements of tocopherols on litter size, on the size and vigor of newly born pigs, and on the growth of these pigs during the nursing and post-weaning periods under normal management conditions.

In the studies to be discussed, the results were complicated by the occurrence of "baby pig disease." This is an ill-defined term, loosely used to describe conditions that result in a high mortality of baby pigs shortly after birth. Because attempts to demonstrate the presence of an infectious agent have been unsuccessful,^{2, 3} it is believed that nutritional factors play an important rôle in the disease.^{4, 5} Recent studies have indicated that the disease may be caused by a virus infection in the dam during the gestation period.^{6, 7}

In the Hormel Foundation swine herd, on which the present studies were made, the breeding is so managed that, on the average, a litter of pigs is born every day throughout the year. Under these conditions, despite careful sanitary practices, "baby pig disease" is enzootic in the herd and, at various times, breaks out in epizootic form. Whether or not this is an unfortunate complication in the present experiments, data have been gathered concerning the effect of dietary tocopherols on the mortality of baby pigs. The investigation was extended to include the effect of dietary tocopherols on the keeping time of body fats.

Results and Discussion

The diets used in the investigation are shown in TABLE 1. The gestation diet, although not devoid of tocopherols, contains a lower level than is likely to be fed under most practical swine-feeding programs. The inclusion of

* Hormel Institute publication No. 40.

TABLE 1
DIETS

<i>Ingredients</i>	<i>Gestation period</i>	<i>Lactation period</i>	<i>Growing period</i>
Ground yellow corn	70	44	41
Ground oats	10	7	20
Dried skim milk	6	2	
Meat scraps			5
Tankage	6	8	5
Soybean oil meal	5	6	7
Linseed meal		4	
Bran		6	
Wheat middlings			10
Alfalfa leaf meal (dehydrated)		20	10
Bone meal	1	1	
Salt	1	1	
Commercial mineral			2
Cod liver oil*	0.25	0.25	
Estimated vitamin E content, mg./lb.†	13	44	31

* 400 USP units of vitamin D and 2000 USP units of vitamin A per gram.

† Calculations based on values of Ellis and Madsen, U.S.D.A., A.H.D. 61, 1943.

alfalfa meal, as in the lactation and growing-period diets, improves the diet and also increases its tocopherol content to an extent that depends on the quality of the meal.

All the diets were fed under dry-lot conditions. The gestation diet was hand-fed at the rate of 6–7 lb. per day, depending on the condition of the animals. The diets used during the lactation and growing periods were self-fed and were the same as those used routinely in the feeding program of the Hormel Foundation.

The experimental animals were gilts of approximately the same age and weight, randomly selected from the Hormel Foundation herd. When gilts weighing about 200 lb. exhibited a heat period, they were removed from the herd and placed alternately in two experimental lots. The animals in group I served as controls. The animals in group II received daily supplements with the morning feed of 1 gm. of mixed tocopherols per gilt, in the form of a 5 per cent mixed tocopherol concentrate.* This supplement increased the tocopherol intake of the gilts in group II to about ten times that of the control group.

All gilts were bred in the first heat period occurring after they were placed in the experimental lots. The gilts in group II had received a total of from 18 to 30 grams each of mixed tocopherols prior to breeding.

Groups I and II originally contained 12 and 13 gilts respectively. Five animals from each group either failed to conceive on the first or second service, or failed to exhibit a second heat period. The reproduction and lactation performances of the gilts that conceived are shown in TABLE 2. The seven control gilts of group I farrowed a total of 66 live pigs, or an average of 9.4 pigs per litter, as compared with 66 pigs farrowed by the eight gilts in group II, an average of 8.1 pigs per litter. Since the ex-

* Supplied through the courtesy of Distillation Products, Inc., Rochester, New York.

TABLE 2
FARROWING AND WEANING RECORDS

<i>Sow no.</i>	<i>Pigs born alive</i>	<i>Average pig weight</i>	<i>Number weaned</i>	<i>Average weaning weight</i>
Group I—Control diet*				
1	11	2.2 lb.	2	21 lb.
2	9	3.3	4	23
3	14	2.5	5	19.5
4	10	2.3	0	—
5	3	3.0	1	16
6	14	2.8	2	36
7	6	3.6	1	17
Average per litter	9.4	2.75	2.1	22
Group II—Supplemented Diet†				
8	9	2.6	6	31.5
9	13	3.0	9	23.5
10	5	4.0	5	20.5
11	9	1.9	5	26.0
12	6	3.0	6	31.0
13	10	3.0	0	—
14	7	2.9	5	27.5
15	6	3.0	5	24
Average per litter	8.1	2.86	5.1	26

* Dams farrowed between November 3 and December 2, 1948.

† Dams farrowed between October 2 and November 14, 1948.

perimental groups were small, these data permit no interpretations concerning possible effects of tocopherols on litter size. The dietary tocopherols appeared to have no effect on the size of pigs at birth. In general, all dams apparently farrowed healthy pigs of normal size.

The pigs in group I had a higher mortality rate during the 56-day nursing period than the pigs in group II: 51, or 77 per cent of the pigs in group I, and only 24, or 37 per cent of those in group II died. In both groups, the period of highest mortality was the first week of life. The pigs manifested the typical symptoms described for "baby pig disease." As far as can be ascertained, the dams and the pigs from both groups were equally exposed to the somewhat uncertain causes of "baby pig disease." The mortality differences between the two groups, therefore, may apparently be attributed to effects of the dietary tocopherols. Until we have conducted further experiments, however, we cannot rule out the possibility that the control dams and their pigs were exposed to the causes of "baby pig disease" to a greater extent than the dams receiving the tocopherol supplement.

The pigs farrowed by the control dams made poorer gains during the nursing period than did the pigs farrowed by the tocopherol-supplemented animals. The average weight of the pigs at 56 days was 22 lb. for the control group and 26 lb. for the supplemented group. Again, we believe that the faster gains were probably due to the influence of the tocopherol supplement. More directly, they may be due to the favorable effect of tocopherols on the quality of the milk produced by the dams in group II.

At weaning time, the pigs from the control dams were evenly divided into two groups (III and IV), according to weight and sex. Pigs in group III received the growing-pig diet shown in TABLE 1, and those in group IV received the same diet plus a daily supplement of 0.25 gm. of mixed tocopherols* per pig for the first six weeks and 0.5 gm. per pig thereafter. The pigs weaned by dams in group II were similarly separated into groups V and VI and were placed on the same dietary regimes, respectively, as the pigs in groups III and IV.

The growth data for the weaned pigs are given in TABLE 3. These data

TABLE 3
AVERAGE WEIGHT OF PIGS AFTER WEANING*

<i>Time</i>	III <i>No supplement</i>	IV <i>Supplement to pigs</i>	V <i>Supplement to dams</i>	VI <i>Supplement to dams and pigs</i>
0 weeks	25.7 lb.	22.6 lb.	27.5 lb.	29.6 lb.
5 weeks	36.7	31.3	38.0	41.9
10 weeks	56.1 (6)	47.3 (7)	61.8 (13)	67.4 (14)

* The data are averages for only those pigs that survived for 10 weeks after weaning. The figures in parentheses represent the number of pigs surviving in each group.

are somewhat difficult to interpret because some pigs died from causes that were in no way related to the diet. The table includes data only for those pigs that survived and were gaining weight at the end of the 10-week period. The weight advantage of the pigs in groups V and VI over that of the pigs in groups III and IV appears to be a reflection of the health and vigor of the pigs during the lactation period, the greater gains being attributable to the tocopherol supplement received by the dams during gestation and lactation. The tocopherol supplements fed to the weaned pigs in groups IV and VI had no demonstrable effect on the growth during the post-weaning period.

Barnes and coworkers,⁸ investigating the effect of dietary tocopherols on the keeping time of the body fats, found that the induction period of the fats of rats that were fed a normal diet containing appreciable amounts of tocopherols could not be significantly increased by dietary supplements of tocopherols. However, the keeping time of body fats of rats fed diets low in tocopherols was markedly reduced, and supplementation of the diet with tocopherols restored the fats to normal keeping times. Watts and coworkers⁹ investigated the effect of dietary tocopherols on the keeping time of body fats of swine and found that tocopherol supplementation did not improve the stability of hog fats.

In view of the apparent species difference noted in the above findings, it seemed desirable to study the keeping time of body fats of the pigs used in the present investigation. For this study, two pigs from each group were slaughtered about 13 weeks after weaning. Fat tissues from the back, leaf, and mesentery were taken immediately after slaughter. The tissues

* Supplied through the courtesy of Mathews Supplements, Rochester, N. Y.

from the four pigs from groups V and VI were rendered by heating under vacuum in a water bath at 80° C. for about 5 hours and at 100° for one hour. The tissues from the pigs in groups III and IV were rendered by heating in a water bath at 100° C. for one-half hour and then under vacuum for one and one-half hours. All samples were filtered once through muslin and twice through a thin layer of anhydrous Na_2SO_4 .

The average keeping times of the body fats from the four groups of weaned pigs are given in TABLE 4. The iodine values (Wijs, 30 min.) were quite

TABLE 4
AVERAGE KEEPING TIME OF FATS*

	III <i>No supple- ment</i>	IV <i>Supplement to pigs†</i>	V <i>Supplement to dam</i>	VI <i>Supplement to dam and pigs†</i>
Induction period in minutes				
(Oxygen absorption method)				
Back fat	17	50	9	58
Leaf fat	37	73	17	79
Ruffle fat	9	69	30	83
Induction period in hours				
(Active oxygen method)				
Back fat	1.5	5.5	—	—
Leaf fat	6	14	—	—
Iodine value				
Back fat	80.07	80.69	81.53	82.90
Leaf fat	70.05	70.84	72.13	72.61
Ruffle fat	57.14	57.14	52.79	57.72

* All results are averages of individual values from fats of two pigs.

† Daily supplement to pigs of 0.25 gm. of mixed tocopherols for first six weeks post-weaning and then 0.5 gm. per day for about six weeks.

constant for each type of tissue fat. In each case, the induction period, as measured by an oxygen absorption method at 100° C., of the fats from pigs that did not receive supplemental tocopherols during the post-weaning period was much shorter than the induction period of the fats from pigs fed supplementary tocopherols (groups IV and VI). The active oxygen method was used to determine the induction period of back and leaf fats from the pigs in groups III and IV. The results by this method corroborate the results of the oxygen absorption method.

From these results, it is apparent that the stability of hog fats can be influenced by the diet. Chipault and coworkers¹⁰ have demonstrated that, in general, the keeping time of hog fats is dependent primarily on their composition and tocopherol content. Since all pigs were fed the same diet, the nearly constant iodine values of the fats obtained from any one tissue indicate similar fatty acid compositions. The longer induction periods of the fats from hogs fed supplemental tocopherols are, therefore, a qualitative

measure of the amounts of tocopherol stored in the tissues. The tocopherol contents of the fats from hogs fed tocopherol supplements were two to three times greater than the tocopherol contents of fats from hogs fed a normal diet.

The discrepancies between our findings and those of Watts and coworkers⁹ can undoubtedly be explained by the differences in the experimental procedures employed. The fats tested by Watts and coworkers were derived from pigs fed much smaller amounts of tocopherols or from pigs fed over a much shorter period. The fats studied in this investigation were taken from pigs fed tocopherols over a 12-week period, and the amount of mixed tocopherols ingested per pig during that period was about 31 grams.

Summary

It appears that supplementation of the diet of the dams with tocopherols during the gestation period did not affect the size or apparent health of the pigs at birth but did favorably affect the livability and the growth of the nursing pigs under environmental conditions in which so-called "baby pig disease" is enzootic.

Supplementation of the pig diets with tocopherols during the post-weaning period did not affect the growth of the pigs, regardless of whether the pigs had been farrowed by dams that had received tocopherol supplements.

The stores of tocopherols in the body fats of the pigs that received no supplement of tocopherols during the post-weaning period were at about the same level, regardless of the dietary regime of their dams. However, supplementation during the post-weaning period did increase the stores of tocopherols in the body fats.

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Discussion of the Paper

EVAN SHUTE (*Department of Medicine, The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): What results were obtained from the electrocardiographic studies made on the vitamin E-deficient pigs?

WALTER LUNDBERG: Dr. Essex of the Mayo Clinic has reserved judgment until he can study the EKG's more carefully.

HANS KAUNITZ (*Departments of Pathology and Animal Care, College of Physicians and Surgeons, Columbia University, New York, N. Y.*): Was there evidence of virus disease involvement in the experimental pigs?

WALTER LUNDBERG: Dr. Young of the University of Minnesota believes he has shown that the dams appear to be infected with a virus which sensitizes the piglet *in utero* in a manner comparable to Rh incompatibility.

THE TOCOPHEROL SERUM LEVEL OF COWS AND HORSES IN RELATION TO REPRODUCTION

By F. C. van der Kaay, G. H. B. Teunissen, A. Emmerie, and M. van Eekelen

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Introduction

According to Herschel,³ Vogt-Møller,⁵ and Moussu,² vitamin E therapy has a favourable effect in sterility of the farm animals, cows, and horses. Herschel in Holland and Vogt-Møller in Denmark found vitamin E useful and obtained good results in 75 per cent of their cases. In France, Moussu could reduce the sterility in cows, caused mainly by *Brucella* infection, to two per cent by vitamin E therapy.

Sterility in cows and, to a lesser extent, in horses also is an economic problem, and any effort towards the solution of this problem would save both time and labour. The eventual significance of vitamin E in this sterility justified an investigation on a rather big scale. As the clinical observations gave conflicting results, the only possible way to obtain more exact data was to use biochemical determinations.

The determination of tocopherol in blood serum should give a rather concrete picture of the rôle of vitamin E in relation to reproduction. It was not very far-fetched to suppose that an absorption, excretion, or metabolic abnormality of vitamin E should give an abnormal tocopherol level in blood serum.

At the time we started this investigation nothing was known about the tocopherol content of cow and horse serum. The study was carried out in Holland during war and was extended over a period of two years. Eighteen farms and the farm of the veterinary department of the university at Utrecht gave their full cooperation. The tocopherol determinations were carried out according to the method of Emmerie and Engel.¹

Tocopherol Serum Level of Normal Cows and Horses

It soon turned out that the tocopherol serum level in cows was not constant and showed marked differences at a number of farms. The determinations were therefore continued and repeated monthly several times for more than a year in 19 farms. In FIGURE 1 the results are given for a number of cows in one stable; the others (17 in all) gave similar results.

In FIGURE 2 some results are given for one stable (veterinary school), where the animals were put up and kept indoors summer and winter.

From these figures, the striking influence of nutrition on the serum tocopherol level in cows may be seen. In summer, the tocopherol content in cows at pasture increased to about 800 $\mu\text{g.}/100\text{ ml.}$ In winter, the tocopherol level decreased to 100–200 $\mu\text{g.}/100\text{ ml.}$ In the cows of FIGURE 2, no rise in the tocopherol level was observed in the same time and, in winter, tocopherol levels of practically zero were noticed.

The winter rations of the cows belonging to the veterinary school were rather poor at the time and consisted mainly of hay, beets, straw, and meal of

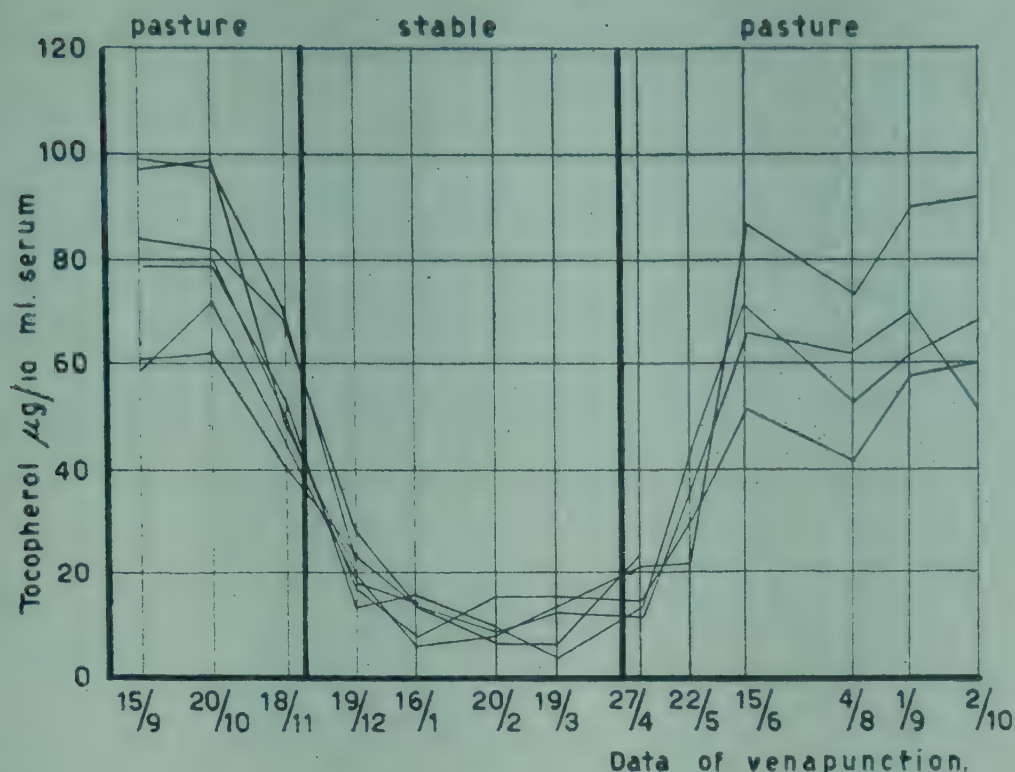


FIGURE 1.

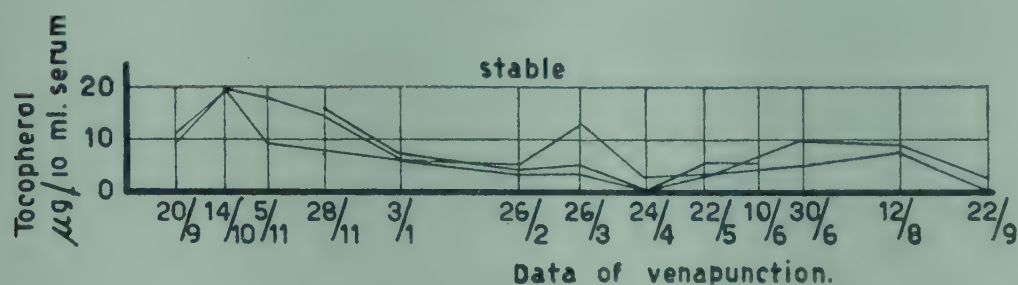


FIGURE 2.

bad quality. In comparison with grass, the tocopherol contents of these components were low (TABLE 1).

TABLE 1
TOCOPHEROL CONTENTS OF SOME FEEDS

Substance	mg. tocopherol/kg.
Grass	60-100
Hay	7-14
Rye and wheat straw	0
Beets	1.8
Meal	11.0

In cows at pasture the intake will be about 2 to 4 grams of tocopherol daily. For the rations consumed during winter no accurate calculations could be made.

In regard to the rapid decrease of the tocopherol level after summer, FIGURE 3 is interesting. In a single cow, the tocopherol determinations were carried out daily after the cow was placed on the winter feed. It turned out that after the first week the tocopherol level had already decreased to about 200 $\mu\text{g}/100 \text{ ml}$. This is of special interest, compared with a similar experiment in man. If a daily dose of 15 mg. tocopherol is given during some weeks, the tocopherol level reaches its maximum after two weeks and remains constant for some weeks when the tocopherol dose is

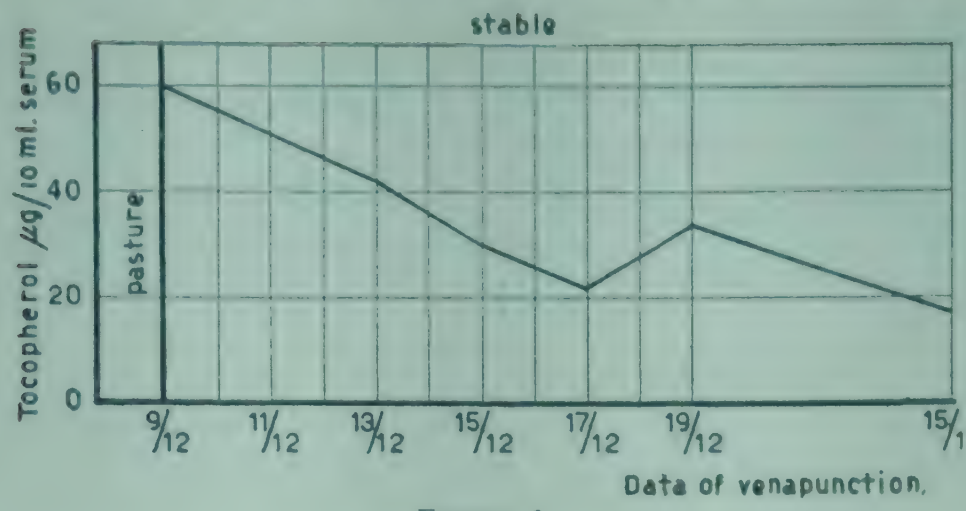


FIGURE 3.

withdrawn. We expected that cows, after summer, would be saturated with tocopherol and that the decrease should progress very slowly in winter, as happens in man.

In FIGURE 4 are given the results of the determinations in normal horses.

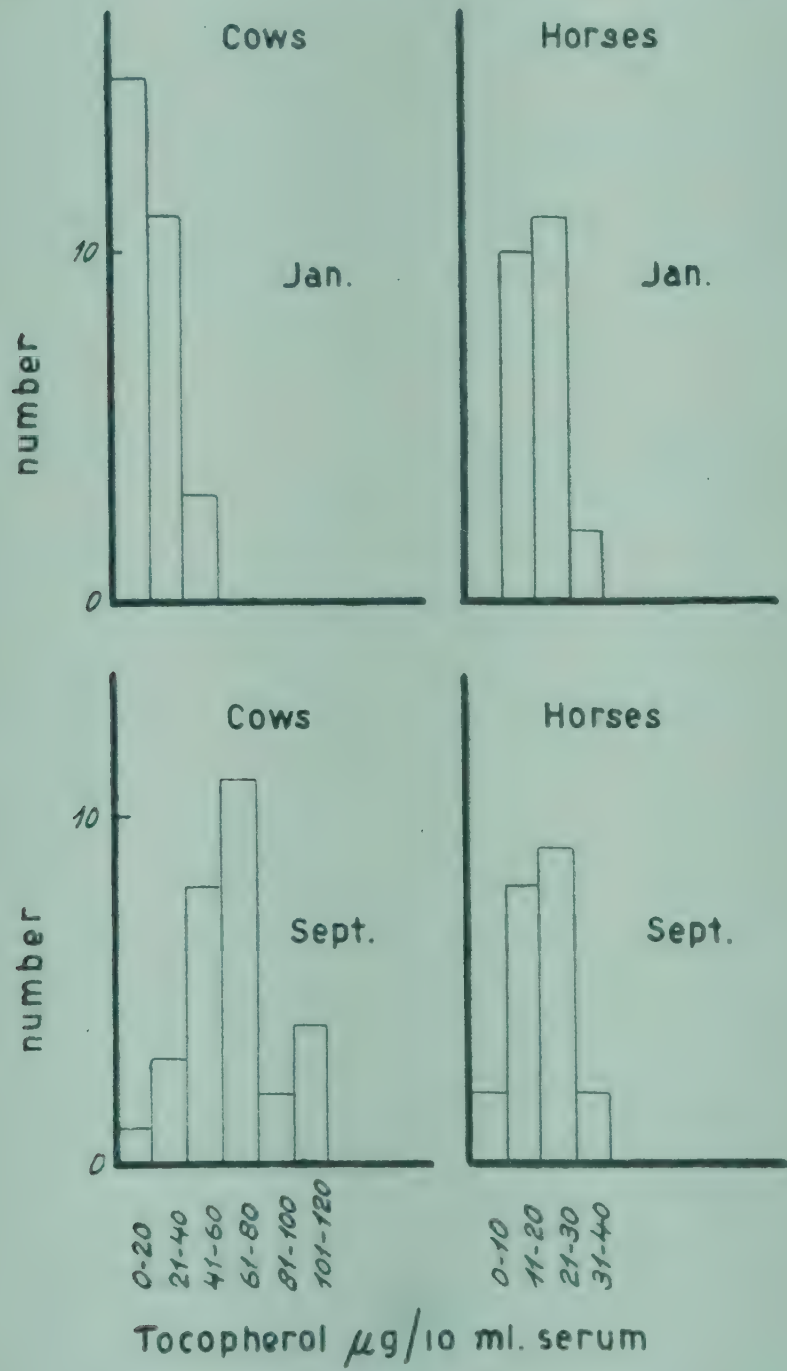


FIGURE 4.

In these animals, living under the same conditions as the cows, no rise in serum tocopherol was observed. The tocopherol values were much lower and were comparable with the levels observed in cows during barn feeding.

The values were almost constant and independent of summer and winter feeding.

In FIGURE 5 the statistical spreading of the values obtained in September and January is given for cows and horses.

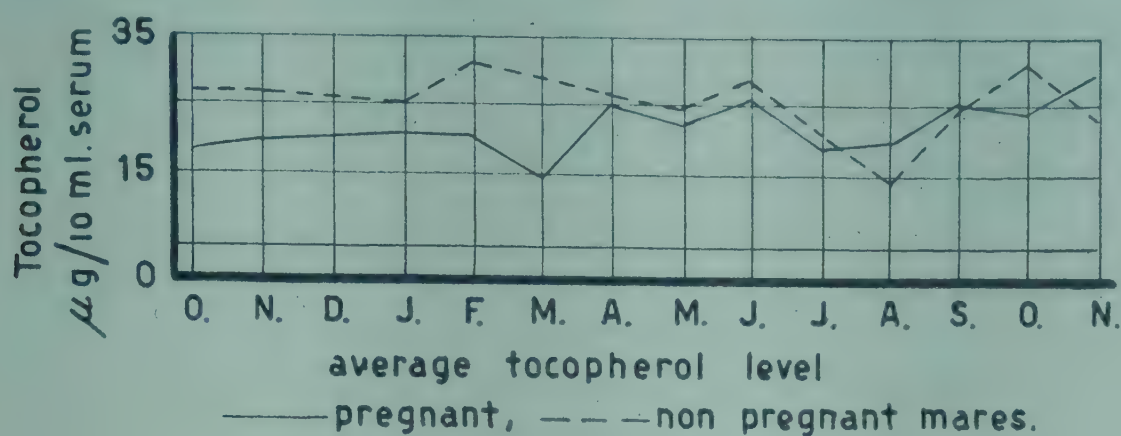


FIGURE 5.

The results so far obtained being unexpectedly complicated by nutrition, we investigated the course of the tocopherol level in the different phases of reproduction: pregnancy, parturition, and lactation.

In FIGURE 6 results have been summarized for 37 pregnant and 11 non-pregnant cows divided equally over different farms.

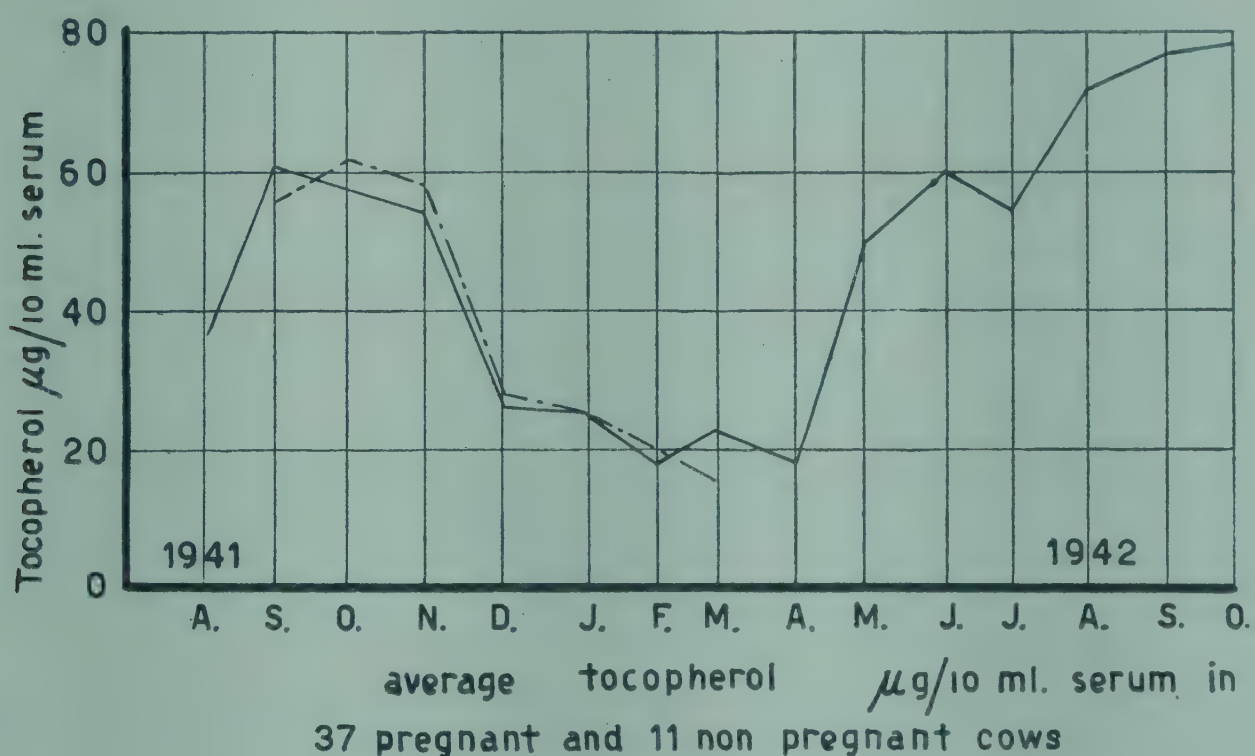


FIGURE 6.

In TABLE 2 are given the results of the determinations before and after parturition. Although there seemed to be some changes at parturition, these were both positive and negative. The average values are therefore the same, and no relation between parturition and serum tocopherol could be observed. The tocopherol level was also determined in some cows during parturition; no increase or decrease was noted.

In the same table the values in relation to lactation are given. No definite change was observed.

The tocopherol level in cows has been related to age. This relation is given in FIGURE 7. Cows selected according to age (one group younger than

TABLE 2

TOCOPHEROL LEVEL OF SERUM BEFORE AND AFTER PARTURITION AND DURING AND AFTER LACTATION

Tocopherol $\mu\text{g.}/10\text{ ml.}$				
before	after		during	after
partus			lactation	
15	33		35	28
18	15		21	18
47	72		33	13
32	23		45	28
54	50		38	20
70	64		16	48
57	64		10	23
32	27		15	27
14	31		26	35
21	22		16	32
10	4		50	67
17	19		56	59
39	28		47	51
68	70		47	54
61	48		13	9
81	64		8	15
51	40		13	6
60	47		6	8
9	42		13	20
			34	9
			13	17
			19	22
			33	27
			34	32
			40	23
			28	10
756	763	Average	739	671
39.7	40.1		28.4	25.8

4 years and a second group older than 4 years) have been compared and a slight correlation is found. The older animals showed a somewhat lower level and were unable to restore their values in summer to the same level as the younger animals.

Tocopherol Serum Level in Brucella abortus Bang Infection, Sterility, Nymphomania and Anaphrodisia.

After these representative determinations in normal cows, we knew exactly how to arrange the determinations in abnormal conditions. For every abnormal case it was necessary to take a comparable normal animal of the same age and the same nutritional status. Lactation and pregnancy were not important. In TABLE 3 the relationship between tocopherol level and *Brucella* infection is given. It may be seen that, in 42 cows with positive agglutination reaction, no abortion occurred, and that the tocopherol level is exactly the same as in 20 cows with a negative reaction. This is a contradiction of a more recent investigation by Scherer.⁴ He carried out a similar investigation and nearly always found a lower tocopherol level in cows with *Brucella* infection.

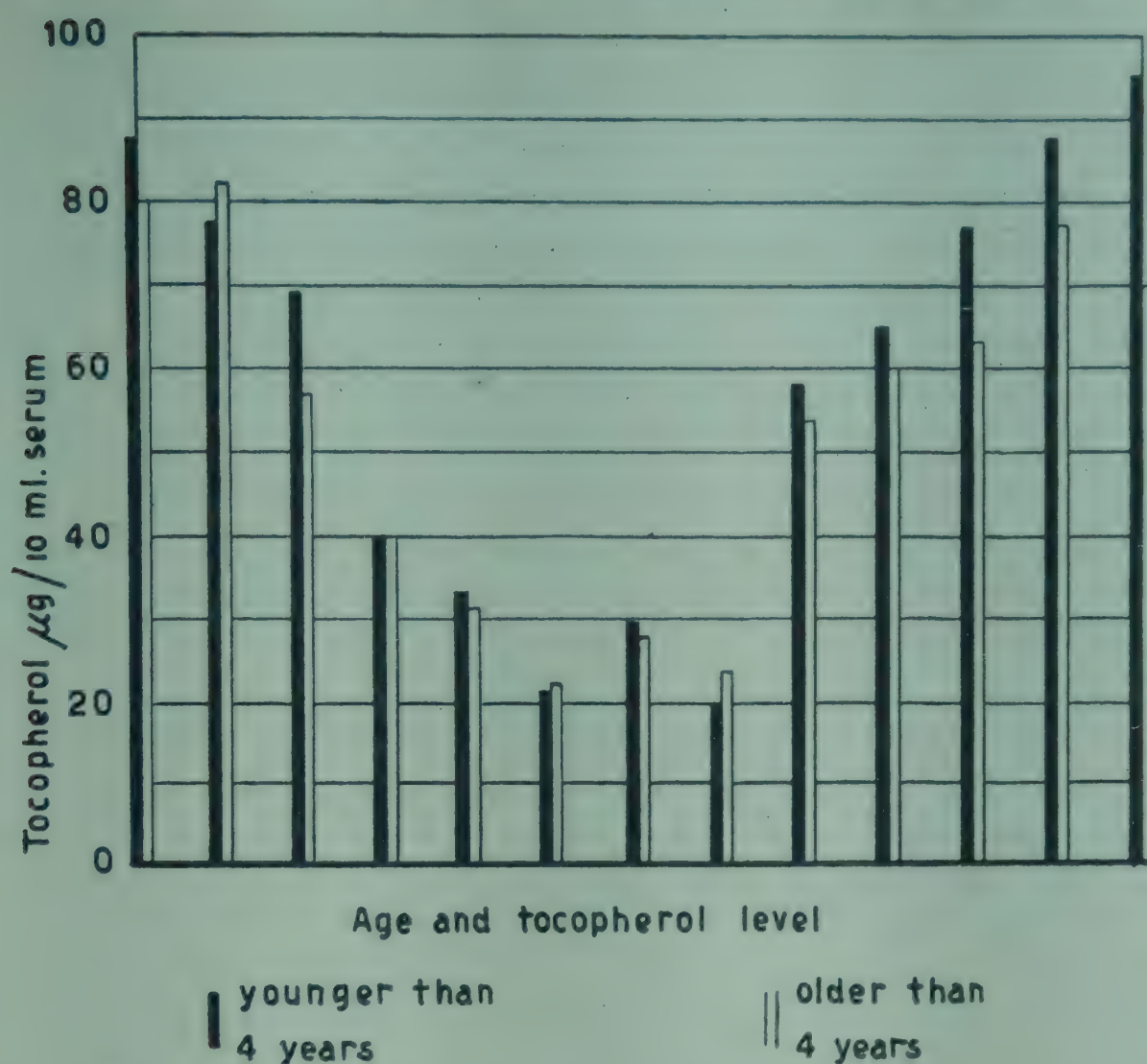


FIGURE 7. Solid bars represent cows younger than 4 yrs. Clear bars represent cows older than 4 yrs.

TABLE 3
TOCOPHEROL SERUM LEVEL IN COWS. *Brucella* BANG INFECTION AND ABORTION

Farm	Farm average	No abortion Bang +	No abortion Bang -	Abortion within 14 d.	Abortion longer than 14 d.
Total number of animals		42	20	22	16
I	30	27	—	22	37
II	13	14	26	13	—
III	25	30	23	20	27
IV	25	29	43	19	—
V	13	14	—	10	—
VI	11	9	10	—	14
VII	17	15	—	14	23
VIII	14	18	17	10	—
IX	25	27	—	27	28
X	10	10	—	9	12
XI	11	11	—	11	—
XII	15	17	—	13	16
XIII	16	11	—	20	18
XIV	18	25	—	14	15
XV	38	40	—	35	—
XVI	9	11	11	6	—
XVII	28	32	—	24	—
XVIII	42	54	—	32	41
XIX	25	25	26	10	17
Average	19	22	22	17	21
	—	—	—	—	—

If abortion had taken place (22 animals), the average of the determinations was somewhat lower but not enough to be significant.

The determination carried out a fortnight (or later) after the abortion gave the normal value. From these results we may conclude—and in contrast with the results of Scherer—that there is no influence of abortion or *Brucella abortus* Bang infection on the tocopherol level of cows' sera.

Sterility of Unknown Origin. In 24 farms, 67 sterile animals and 43 fertile animals were investigated. The fertile animals were divided over these 24 stables in such a way that we always had comparable check animals. The average tocopherol serum level in the 43 fertile cows was 74.1 $\mu\text{g.}/10\text{ ml.}$ and, in the sterile cows, 70.5 $\mu\text{g.}/10\text{ ml.}$ No difference was to be observed.

Nymphomania. In 5 nymphomaniac cows, the tocopherol levels were compared with normal controls. The averages were 810 and 790 $\mu\text{g.}/100\text{ ml.}$ respectively, which difference is not significant.

Anaphrodisia. In 16 young animals, no oestrus was observed. The average tocopherol level was normal.

Sterile Mares. In 18 sterile mares, the tocopherol level of the blood serum did not differ from comparable check animals.

Serum Tocopherol Level in Cows After Administration of Wheat-Germ Oil

Wheat-germ oil was administered in such quantities as to be comparable with the commonly used therapeutical dose. Some of these were believed to have a beneficial effect.

Administration per os. In TABLE 4 is given the daily tocopherol level per

TABLE 4
TOCOPHEROL SERUM LEVEL IN A COW AFTER ORAL ADMINISTRATION OF WHEAT GERM OIL

Dates	Dose administered	$\mu\text{g.}/10\text{ ml.}$
7/12	100 ml. = 226 mg. tocopherol	13
9		16
11		13
13	100 ml. = 226 mg.	14
15	100 ml. = 226 mg.	15
17	300 ml. = 678 mg.	14
18		16

cow after the oral administration of 100 ml. wheat-germ oil = 226 mg. tocopherol.

Intramuscular Administration. The following experiment, carried out with four animals, demonstrates the effect of one intramuscular injection of 25 ml. wheat-germ oil, containing about 80 mg. of tocopherol.

Dates	30/3	31/3*	1/4	2/4	3/4	4/4	5/4	6/4	7/4	8/4
Average of 4 animals $\mu\text{g.}/10\text{ ml.}$	5	5.5	7	8	8	7	6.5	6	6	6

* Injection on 31/3.

Only a slight increase was observed. It is questionable whether these changes in tocopherol level surpass those noted without any administra-

tion. Daily injection of 40 mg. tocopherol for 23 days in a single cow did not increase the blood level significantly.

Intertracheal Injection. In this method of administration the same result was observed. The low dose is probably responsible for this failure to bring about a significant increase in the serum tocopherol level. It may be recalled that the normal intakes from grass are of the order of grams of tocopherol.

Summary and Conclusions

The tocopherol level in normal cows was not constant. It depends greatly on nutrition. In grass feeding (summer), the level is about 800 $\mu\text{g.}/100$ ml. of serum. In winter, 100–200 $\mu\text{g.}/100$ ml. is found.

This level is independent of pregnancy, parturition, and lactation.

There is a slight influence of the age of the animals (probably of nutritional origin).

Cows aborting from *Brucella* infections showed no particularly low tocopherol level. There is no reason to assume a vitamin E deficiency in these cases. Cows with *Brucella* infection but not aborting had a normal level.

In sterile, nymphomaniac, and anaphroditic cows the tocopherol content was normal.

The administration of tocopherol, *per os*, intramuscularly, or intertracheally, usually given in veterinarian practice, did not cause a rise of the tocopherol value of blood serum.

In pregnant and non-pregnant mares, the tocopherol content of the blood serum was much more constant and independent of summer and winter feeding. In sterile horses the value was the same.

Although this investigation has given us a clear picture of the tocopherol level in blood under various conditions, no relation between this level and sterility, occurring in farm animals, was observed.

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STUDIES ON VITAMIN E DEFICIENCY IN THE MONKEY*

By Lloyd J. Filer, Jr., Ruth E. Rumery, Paul N. G. Yu, and Karl E. Mason

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Introduction

Vitamin E deficiency in the *Macaca rhesus* monkey was described by Mason and Telford in 1947.¹ These investigators observed histopathological changes in the smooth muscle and striated muscle of the *Macaca rhesus* monkey comparable to the changes found in other species such as the rat, rabbit, and hamster. The following experiment is a continuation of this earlier investigation, with efforts directed toward a more comprehensive study of the *Macaca rhesus* monkey during the development of chronic vitamin E deficiency and its possible reversibility through vitamin E therapeutics.

Experimental

Animals and Diet. Young monkeys, four to five months old, were obtained from the laboratories of Dr. Gertrude van Wagenen of Yale University. These monkeys were free of tuberculosis, and all possible precautions were taken to maintain them so during the course of this experiment. The animals were purchased in two groups of four each, with a lapse of one year between the groups. Each group of four monkeys was adapted to the basal diet and two of the four were arbitrarily selected to serve as control animals, *i.e.*, to be fed vitamin E (35 mg. mixed natural tocopherols) daily. The animals were housed two in a cage measuring 3' x 3' x 5' and equipped with a drinking fountain and wooden perch.

All monkeys were fed a low fat, vitamin E-deficient diet, having the composition shown in TABLE 1. This mixture of cooked rice and dry ingredients was readily molded into a ball and fed twice daily. Vitamin supplements of A and D or A, D, and E† were given daily in the form of a concentrate spread on a sugar cube. Ascorbic acid (25 mg.) was fed daily.

Throughout the first two years of the experiment, a series of albino rats were fed the same diet. Breeding performances and histopathological studies of these animals indicated that male rats developed testicular degeneration at about 150 days of experiment. Female rats did not experience resorption gestations but showed early lactation failure. These tests indicated the presence of small traces of vitamin E in the diet. Since a state of chronic vitamin E deficiency in the monkeys was desired for the purpose of this study, especially if the animals were to be maintained for subsequent study of reproductive functions, no effort was made further to reduce its vitamin E content or to increase its fat content to accentuate the deficiency state.

* This study is supported by Nutrition Foundation, Inc., New York City.

† Vitamins A, D, and E were supplied in concentrate form by Distillation Products, Inc., Rochester, N. Y.

TABLE 1
BASAL DIET FOR MONKEY EXPERIMENT

<i>Dry Mixture</i>	<i>Grams</i>
Casein	220
Yeast	400
Cerelose	345
Salts	35
Liver Concentrate Powder 1-20	30
Ruffex	70

Add 300 grams dry mix to 1 kilo. of cooked polished rice.
Vitamins A (5000 IU), D (500 IU), and C (25 mg.) fed daily.
Vitamin E (35 mg.) as mixed natural tocopherols fed daily.

Results

Growth. Weekly weighings of all monkeys were made during the early growth phase. The gain in weight per day indicated in TABLE 2 shows

TABLE 2
GROWTH RESPONSE

<i>No. of monkeys</i>	<i>Type of diet</i>	<i>Sex</i>	<i>Weight gain gms./day</i>
4	+E	♀	4.52
2	-E	♀	4.60
2	-E	♂	5.57
5*	Wisconsin M-3	♀	7.3 (4.2-14.8)
7*	Liver 1-20	♂	5.1 (3.3-7.9)

* Ref. 2.

little difference between the two groups and compares favorably with the data of Waisman *et al.*² for a group of monkeys fed the Wisconsin M-3 diet with a supplement of Liver Concentrate Powder 1-20.*

Hematological Studies. Typical results of a routine examination of the blood of all animals for hemoglobin, red cells, white cells, and hematocrit are given in TABLE 3. These data fail to show any differences between the control and deficient groups and are comparable to the hematological data reported for the normal rhesus monkey by Shukers *et al.*³

Blood Glucose and NPN; Plasma Proteins and Tocopherol. On two occasions, blood samples were obtained from the oldest group of monkeys for the analysis of blood glucose and NPN as well as the plasma proteins and plasma tocopherol contents. These data have been summarized in TABLE 4 and, again, fail to show any differences whereby one can separate the deficient animals from the controls other than by the level of plasma tocopherol. The data for blood glucose, blood NPN, and plasma proteins are in good agreement with previously published data for the monkey.^{4, 5, 6}

Electrocardiograms and Pneumocardiograms. Electrocardiograms and

* Liver Powder Concentrate 1-20 was supplied in part by Wilson Laboratories, Chicago, Illinois.

TABLE 3
SUMMARY OF HEMATOLOGICAL FINDINGS

Monkey number	Diet	Length of time on diet: months	R.B.C. millions $\times 10^{-6}$	W.B.C. thousands $\times 10^{-3}$	Hematocrit %	Hemoglobin gms./100 ml.
7	+E	28	6.20	10.6	43	11.7
10	+E	28	5.78	10.45	43	11.6
12	+E	18	5.61	12.95	44	11.3
13	+E	18	5.77	12.05	43	12.0
8	−E	28	5.89	8.05	41	11.6
9	−E	28	5.99	12.85	43	11.9
11	−E	18	5.61	8.30	44	12.6
0	−E	18	4.92	6.35	43	11.8
19 Normal Monkeys ³			5.2+0.6	15.1+5.4	—	12.2+1.3

TABLE 4
BLOOD GLUCOSE, BLOOD NPN, PLASMA TOCOPHEROL, AND PLASMA PROTEIN DATA^{4, 5, 6}

Monkey number	Diet	Length of time on diet: months	Blood NPN mg./100 ml.	Blood glucose mg./100 ml.	Plasma tocopherol mg./100 ml.	Pasma proteins gms./100 ml.
7	E	28	47	72	0.57	6.01
10	E	28	46	80	0.58	6.34
8	−E	28	46	81	0.19	6.55
9	−E	28	34	85	0.17	6.47
Based on Ref. 6.			30–52	—	—	7.25–8.25 (serum)
Based on Refs. 4 and 5.			—	91–140	—	—

pneumocardiograms were recorded during the course of this study on animals anesthetized with nembutal given intraperitoneally at a level of 36 mg. per kilo. body weight. The level of nembutal given the vitamin E-deficient animals was, in some cases, 90 per cent of the calculated anesthesia dose.* A Sanborn Cardiette was used to record the standard three limb leads as well as several unipolar limb and precordial leads of the EKG. Chest and neck pneumocardiograms were recorded simultaneously with the standard EKG lead two, according to the method of Wedd and Blair adapted to the Sanborn Cardiette.⁷ Calculations for the beginning of the ejection time of the heart were determined from the onset of electrical systole and the point of deflection in the chest or neck pneumocardiogram. Three observations have been carried out on each of the four monkeys in the oldest group at 18, 24, and 26 months, respectively. A single observation on each of the four monkeys in the youngest group has been recorded at 14 months of study. The electrocardiographic and pneumocardiographic findings are recorded in FIGURES 1, 2, and 3.

FIGURE 1 indicates that the state of chronic vitamin E deficiency had

* Since initial studies indicated that surgical anesthesia was more rapidly attained in the vitamin E-deficient monkey, the amount of nembutal administered was reduced.

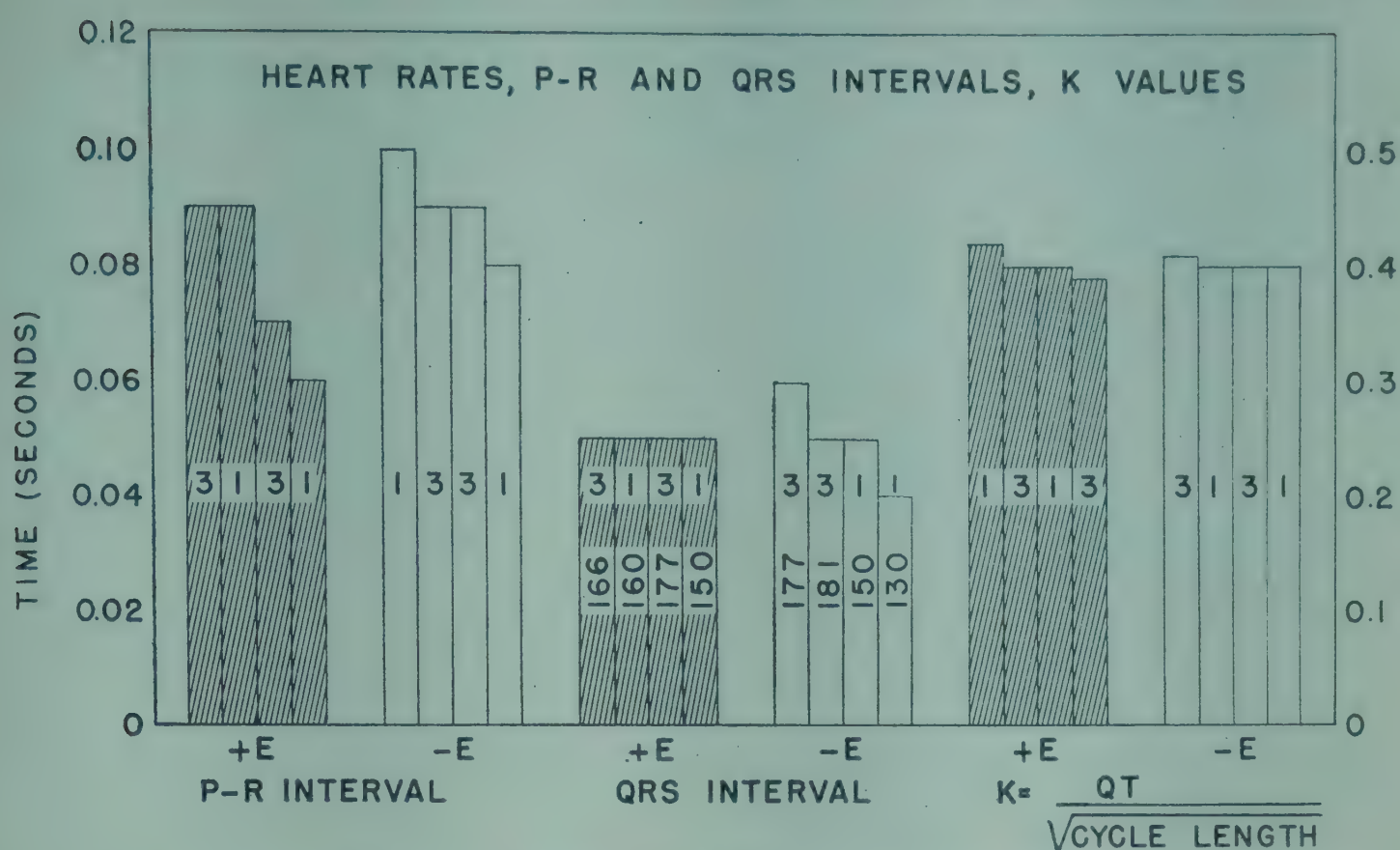


FIGURE 1. Heart rates, P-R and QRS intervals, and K values for control and vitamin E-deficient monkey are represented. The figures within the individual bars indicate the number of observations determining specific value. The heart rates, ranging from 130 to 181 beats per minute, are shown within the bar graph for the QRS interval. The K value, or corrected QT interval, should be read relative to the units on the right of the figure.

little or no effect on the heart rate, P-R interval, QRS interval, or corrected QT interval as recorded in the EKG. No influence on axis deviation was observed. The height of the R waves, shown graphically in FIGURE 2, indicates that the voltage of R_1 and R_2 was higher in the control animals than in the vitamin E-deficient group. These same conditions were found to exist for the R_{V4} and R_{AVL} leads, with the R_3 lead being variable. An analysis of the T waves (FIGURE 2) indicated that the amplitude of T_2 and T_{V4} was greater in the control than in the deficient group, with the T_{AVL} wave being inverted in three of four deficient animals and in only one of four control animals.

The ejection time of the heart, whether determined by chest or neck pneumocardiogram, was always shortened in the deficient animal (FIGURE 3). Clinically, it has been observed by Yu and Bruce that conditions like myocardial infarct or dilatation of the heart due to various causes effectively reduce the isometric contraction phase, as revealed by the earlier initiation of ejection from the ventricles.⁸ The order of magnitude of reduction seen clinically compares favorably with the differences observed between the control and vitamin E-deficient monkey.

Discussion

Macaca rhesus monkeys maintained on a low fat, vitamin E-deficient diet, calculated to induce a chronic deficiency state, show slight but consistent changes in their electrocardiograms and pneumocardiograms compatible with the severity of the deficiency. Other criteria of performance, such as growth, hematological studies, plasma proteins, blood NPN, or blood glucose, fail to differentiate the vitamin E-deficient monkey from the control or normal animal.

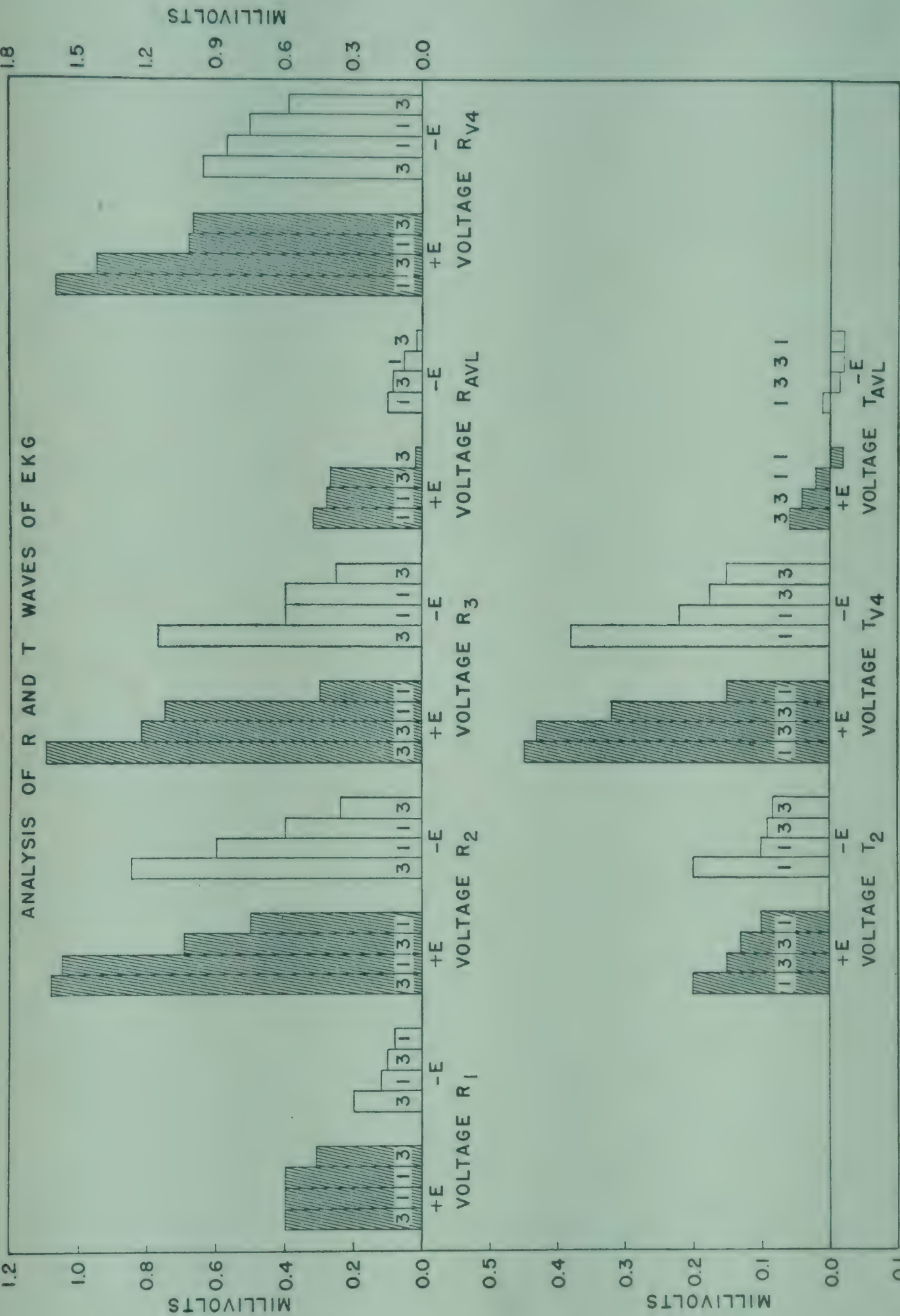


FIGURE 2. The amplitudes of various R and T waves are plotted as millivolt readings. Data for both control and vitamin E-deficient monkeys are shown, with the number of observations specified within or above the bar. R_{V4} should be read relative to the units on the right of the figure.

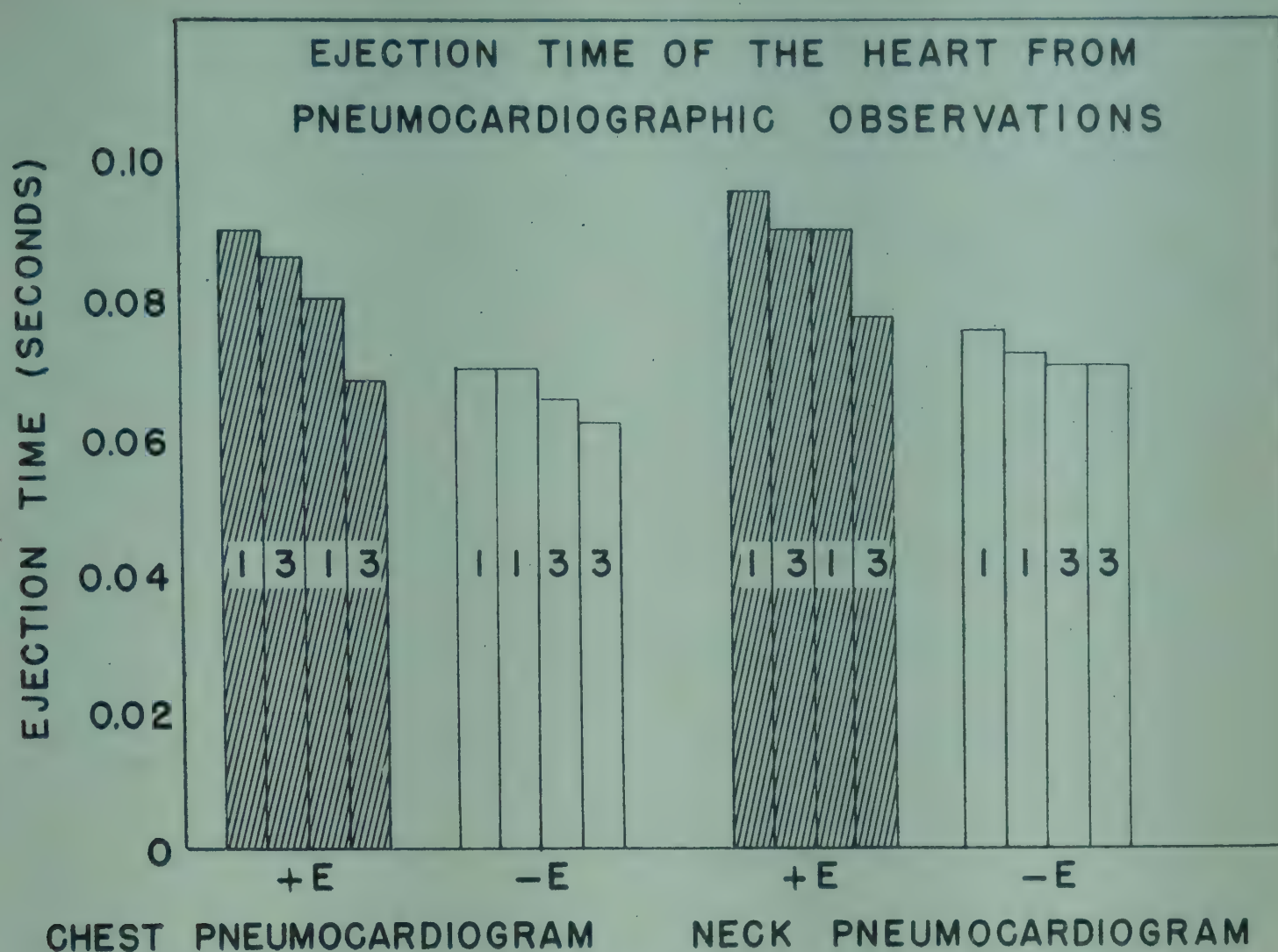


FIGURE 3. The ejection time of the heart, as determined by chest and neck pneumocardiograms, is shown for both control and vitamin E-deficient monkeys. The number of observations determining each specific value is recorded within the bars.

The fact that the diet is low in fat tends to conserve the small quantity of vitamin E available in the basal diet and also minimizes the detectable differences between the deficient and control animal. A relationship between the quantity and degree of unsaturation of ingested fat and vitamin E requirement has been demonstrated in many ways for animals such as the rat, rabbit, and guinea pig and, undoubtedly, is a factor in the present experiment.^{9, 10} Additional evidence substantiating this concept was obtained from the rat-feeding experiments conducted in conjunction with the monkey-feeding experiment.

Electrocardiographic data for the normal *Macaca irus* monkey has been recorded by de Waart and Storm.¹¹ In observations on 12 normal monkeys, the pulse rate ranged from 171 to 261, P-R interval 0.054 to 0.088 sec., and QRS interval from 0.020 to 0.044 sec., while the R₂ wave registered 0.47 to 1.41 millivolts, the ST segment was isopotential, and the T wave was upright and well developed. Ruskin and Rigdon have recently reported data for 14 normal *Macaca rhesus* monkeys, finding heart rates varying from 190 to 280, P-R intervals from 0.06 to 0.10 sec., and QRS intervals from 0.02 to 0.04 sec.¹² These investigators also claimed low voltage in R₁, T₁, T₂, and T₄, as part of the normal EKG pattern of the monkey, and stated that their findings were contrary to the results of de Waart and Storm. However, the results of our study apparently correlate well with the earlier observations of de Waart and Storm.

Electrocardiograms have been recorded for the rat, rabbit, and cow in the vitamin E-deficient state. Ensor reported that rats maintained on a

vitamin E-deficient diet for one year showed little or no change in EKG records, relative to normal controls.¹³ No histopathological evidence of cardiac damage or induced vitamin E deficiency was recorded with these observations. Martin and Faust, in a study of four rabbits, observed a slowing of the heart rate, change in axis deviation, and an increase in T-P and Q-T times, conditions similar to those reported by Gullickson and Calverly for vitamin E-deficient cattle.^{14, 15} Recently, Bragdon and Levine¹⁶ produced acute myocarditis with associated abnormal electrocardiographic changes in the rabbit. In particular, these investigators found an elevation of the S T segment and an inversion of the T wave.

Waisman and McCall have reported¹⁷ EKG changes in thiamine deficiency in the *Macaca mulatta* monkey. Bradycardia, decrease in the height of the R wave, and inversion of the T wave were found. These changes were readily reversible when thiamine was administered to the deficient animals. Equally striking differences between deficient and control animals were observed in our experiment, with further extension of the studies to include unipolar limb and precordial leads as well as measurements of heart ejection times. The unipolar limb and precordial leads indicated some difference between the normal and deficient animal, and the ejection time of the heart was reduced about 13 per cent or more in the case of the chronically E-deficient animal.

It is planned to continue these observations on the monkey until a maximum differential between the deficient and normal animal is reached and, then, instigate vitamin E therapy to determine the degree to which these electrocardiographic and pneumocardiographic changes are reversible.

Summary

A chronic vitamin E deficiency in the *Macaca rhesus* monkey, in conjunction with a low fat diet, leads to slight but consistent changes in the electrocardiogram and pneumocardiogram relative to a control animal. Reduction in the amplitude of the R and T waves, with inversion of the latter and shortening of the time for initiation of ventricular ejection from the heart, are the essential findings. The type of change observed is in accord with EKG studies on other species in vitamin E deficiency and is similar to some of the EKG changes recorded for thiamine-deficient monkeys.

Other criteria, such as growth, hematological studies, blood glucose, blood NPN, and plasma proteins, fail to differentiate the chronic vitamin E-deficient animal from the controls.

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Discussion of the Paper

DR. E. SHUTE (*Department of Medicine, The Shute Institute for Clinical Laboratory Medicine, London, Ontario, Canada*): Were platelet counts carried out on these vitamin E-deficient monkeys?

DR. L. FILER: No.

DR. J. MACKENZIE: Were there any outward manifestations of vitamin E deficiency in the monkeys, such as reduced activity?

DR. L. FILER: No. Outwardly, one could not differentiate the deficient from the control animals.

DR. W. SHUTE (*Department of Medicine, The Shute Institute for Clinical and Laboratory Medicine, London, Ontario, Canada*): Was there any evidence of edema or other lesions in the extremities of the vitamin E-deficient monkeys which could explain the changes in their EKGs.

DR. L. FILER: There was no gross evidence of peripheral edema.

DR. W. GOVIER (*Department of Pharmacology and Endocrinology, The Upjohn Company, Kalamazoo, Mich.*): Since thiamine has been related to EKG changes and Holmes has reported an interrelationship between vitamin E and thiamin, might there not be some influence of the high thiamine intake on the EKG changes which were observed?

DR. K. MASON: The report of Holmes, claiming a beneficial effect of thiamine in late-weaning paralysis of rats, could not be substantiated by studies carried out some years ago by Dr. Roger Terry in my laboratory. Hence, I think that we can rule out possible interactions between thiamine and tocopherol. However, the effect of high thiamine on the EKG of our monkeys has not been tested.

VITAMIN E IN HUMAN NUTRITION

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Introduction

Since the first vitamin E conference held in London ten years ago, we have carried out our researches in various ways. The possibilities for investigating the significance of vitamin E in human nutrition and physiology were rather limited but we applied three kinds of methods: (1) the requirement of vitamin E was studied by determining the dietary intake, the absorption from the intestine, and the excretion; (2) the relation of blood tocopherol level and intake was studied by depletion and supplementation experiments; (3) the therapeutical application of vitamin E in different pathological conditions was studied by comparing the clinical observation with the determination of serum tocopherol content.

Dietary Intakes

At the time we started these investigations, nothing was known about the vitamin E content of foods. A chemical method for determining this vitamin in different foods was developed, and, guided by a number of estimations, we were able to calculate the tocopherol content of an average Dutch diet. This was not only of scientific importance but, also, was highly relevant to practical nutrition. Thus, during World War II, when the diet of the Dutch population changed considerably, we could predict the tocopherol content of this changed diet together with the levels of other, better known, nutrients. Some of the results are given in TABLE 1.

The tocopherol content of the green vegetables which are most commonly used—spinach, endive, and kale—is high. The colorless vegetables, like onions, Belgian endive, beets, and potatoes, are very poor sources of vitamin E. All cereals have a content of the same order. The vitamin E content of the dairy products is low.

The vegetable oils, with the exception of coconut oil and olive oil, show the highest levels. The purified oils, however, as used in consumption, were much poorer. These vegetable oils, the main constituents of margarine, are all import products for the Dutch people. During the war, rape seed, linseed, and poppy-seed oil were used in their place. All these had moderate tocopherol contents.

We also looked for rich sources to be used if necessary as a substitute for wheat germs and wheat-germ oils. Pine needles and seeds of cord grass (*Spartina townsendii*), growing in muddy saltmarshes along the North Sea shores, showed a high level of 15 and 13 mg./100 g., respectively, and were in abundant supply.

As a result of these determinations, we were able to calculate the intake for an average Dutch diet. The calculation is given in TABLE 2.

This diet for adults, with a caloric value of 2600 Calories, has been calculated from several dietary surveys and represents the daily intake of the

TABLE 1
TOCOPHEROL CONTENT OF FOODS IN MG. PER 100 G.⁶

<i>Material</i>	<i>Tocopherol content</i>	<i>Material</i>	<i>Tocopherol content</i>
<i>Vegetables</i>		<i>Cereals</i>	
Beans (kidney)	1.2	Barley	4.2
Beans (white)	4.0	Biscuit	2.4
Beets	0.2	Bread (brown)	2.1
Cabbage (red)	0.2	Bread (white)	1.4
Cabbage (white)	0.7	Groats	1.5
Carrots	1.5	Oats	2.0
Celery	2.6	Rice, polished	0.4
Endive	2.0	Rice, unpolished	2.9
Endive (Belgian)	0.2	<i>Dairy products</i>	
Kale	8.0	Cheese (20% fat)	0.6
Leek	1.9	Cheese (10% fat)	0.3
Lettuce	0.6	Eggs	3.0
Onions	0.2	Meat	0.6
Parsley	5.5	Milk	0.03
Peas (green)	6.0	<i>Oils and fats</i>	
Peas (grey)	8.0	Peanut oil	26.0
Potatoes (cooked)	0.1	Butter	2.6
Spinach	1.7	Cocoa butter	12.5
Sprouts (Brussels)	1.7	Coconut oil	5.0
Turnips	0.02	Olive oil	3.0
		Palm oil	110.0
		Soybean oil	120.0

TABLE 2
AMOUNTS, CALORIC VALUES, AND TOCOPHEROL CONTENTS OF THE CONSTITUENTS OF AN AVERAGE DUTCH DIET⁹

<i>Material</i>	<i>g./day</i>	<i>Caloric value</i>	<i>mg. tocopherol</i>
Meat	50	125	0.30
Fish	15	22	0.15
Egg	10	16	0.30
Milk	350	189	0.10
Cheese	15	51	0.09
Butter	15	118	0.33
Fat	9	84	0.01
Cereal products (except bread)	30	105	1.02
Potatoes	450	414	0.45
Vegetables	182	93	4.60 (8.00)
Fruits	70	27	0.01
Sugar	55	214	—
Various	27	69	—
Margarine and oil	20	153	2.00
Bread (wheat)	400	924	5.60
(Bread (rye 100%))	(300)		(6.00)
		2613	14.96 (14.00)

workers and officials of a moderate income class, living in towns and not doing heavy work. The use of the more expensive foods, meat and eggs, is restricted in this diet. From this table it can be seen that the tocopherol

intake is mainly supplied by vegetables and bread. For vegetables, the intake was calculated on the basis of the average Dutch vegetable consumption. From this calculation, we concluded that the intake was about 15 mg. a day.

Guided by these results, we carried out further investigations in two directions. As the intake was mainly dependent on vegetables and bread, we investigated the absorption of tocopherol from vegetable sources.

From the investigations of van Eekelen *et al.*,⁴ carried out in our laboratory, we knew that the human absorption of the fat-soluble pro-vitamin carotene was poor from cooked vegetables and rather good from oils and aqueous emulsions.

We carried out the same experiment with tocopherol. As the determination of tocopherol in human feces did not yield satisfactory results, rats were used for these absorption experiments. In TABLE 3, the results are

TABLE 3
ABSORPTION OF CAROTENOIDS AND TOCOPHEROL

<i>Material</i>	<i>Carotene absorption in man (according to van Eekelen⁴)</i>	<i>Xanthophyl absorption in man (according to van Eekelen⁴)</i>	<i>Tocopherol absorption in rats</i>
Spinach cooked	6%	32%	13%
Carrots	1%	—	—
Carotene in coconut oil	59%	—	—
Tocopherol in olive oil	—	—	69%
Xanthophyl in peanut oil	—	40%	—

given and compared with absorption experiments of carotene and xanthophyl. From these results we may conclude that tocopherol in cooked vegetables is, probably, only partly used and that the tocopherol intake, therefore, depends mainly on fat and bread consumption. So a second study was undertaken to learn the relation between the composition and tocopherol content of bread.

In collaboration with other laboratories and milling factories, all nutrients, including thiamin, riboflavin, niacin, tocopherol, and trace metals, were determined in the various milling fractions.³

In TABLE 4 and FIGURE 1 are given the results for one of these investigations, using Dutch home-grown "Juliana" wheat. From these data, it was possible to calculate the changes in intake of nutrients caused by any alterations being made in the baker's flour.

The pre-war flour was a mixture of the first three fractions containing 77 per cent of the whole grain and about 48 per cent of the tocopherol. Soon after the beginning of the war, the percentage of extraction had to be changed to 85 per cent. This involved a rise in tocopherol to 68 per cent. Later on, the baker's flour contained a greater percentage of rye, barley, potatoes, and sometimes peas. At the end of the war, the percentage of extraction was gradually raised and the ration was continuously decreased.

TABLE 4
TOCOPHEROL CONTENT OF MILLING FRACTIONS OF WHEAT³

Milling fraction		Extraction %	Tocopherol mg. %	Tocopherol content % of total	
b1	patent flour	31.7	1.8	20.4	48.1
b2	1a flour	31.7-54.7	1.6	13.1	
b3	1b flour	54.7-77.6	1.8	14.7	
b4-b9	middlings	77.6-80.3	8.9	8.6	
c1	white dogs	80.3-81.4	9.6	3.8	
c2	red dogs	81.4-84.5	8.3	9.2	67.7
c3	fine shorts	84.5-84.6	4.3	0.15	
c4	coarse shorts	84.6-90.5	3.0	6.4	
c5	bran	90.5-97.1	2.5	5.9	
k	germs	97.1-98.6	25.6	13.7	
		0-100	2.8		
				96.0	

In the meantime, the vegetable consumption increased two and threefold. In TABLE 2, the figures in brackets account for these changes. Although no accurate figures can be given for the food intake in the last period of the War (Sept., 1944-May, 1945), we may conclude that the tocopherol intake did not seriously decrease.

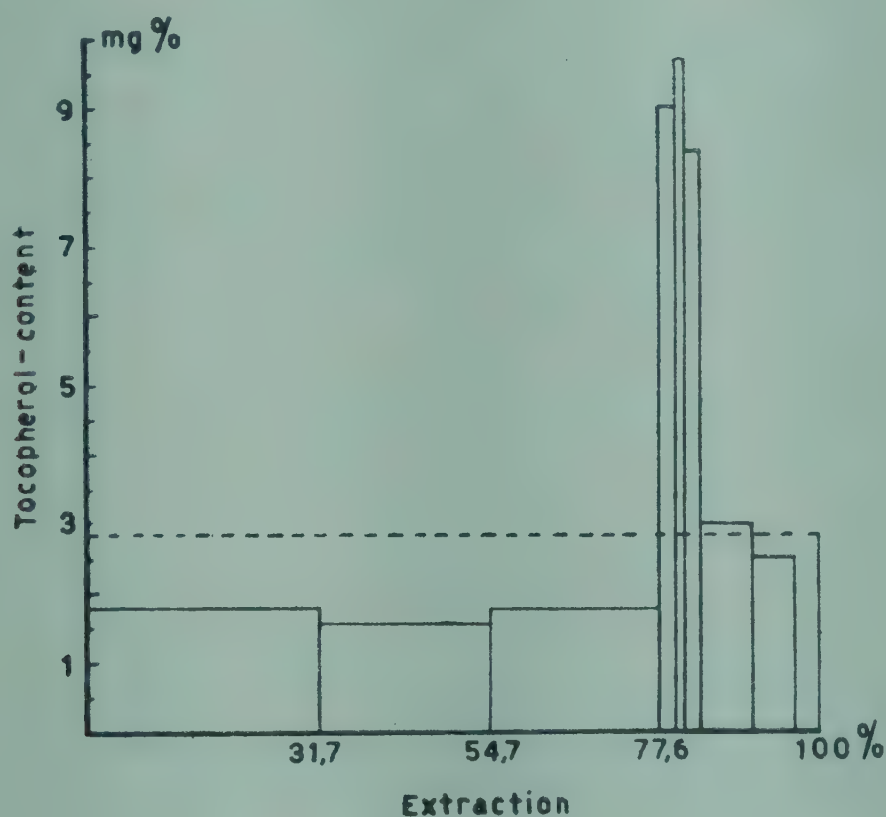


FIGURE 1.

The general conclusion from these dietary studies is that the calculated intake of tocopherol in the average Dutch diet is about 15 mg. a day. The absorption of tocopherol from vegetables being poor, not more than about 10 mg. will actually be available.

The Relation Between Blood Tocopherol Level and Intake

For the method of determination of tocopherol in blood serum, we may refer to our original paper.⁵ We have made some unpublished alterations since, which may be useful for others.

Our rather laborious extraction method was compared with that of Kimble⁷ for vitamin A determinations in blood sera or plasma. The latter method also gave satisfactory results for vitamin E.

For the separation of the tocopherols from interfering substances, we applied a chromatographic procedure using Floridin X S earth as adsorbent. Many others had difficulties when using this adsorbent. It turned out, however, that a mixture of silicagel and 80 per cent sulfuric acid (10 g. dried silicagel sieved to 30–50 mesh and 5 ml. 80 per cent sulfuric acid) was very satisfactory and could be prepared in a very reproducible way.

To establish the relation between tocopherol intake and serum level, depletion and supplementation experiments were carried out.

Depletion Experiments with Rats. Rats with serum tocopherol content of 200 $\mu\text{g.}/100\text{ ml.}$ were kept on a vitamin E-deficient diet. After definite times, the fertility of the females and males was tested by mating with normal rats. After 70 to 90 days, 70 per cent of the males and females were sterile. The tocopherol level of the blood serum was then decreased to 50 to 70 $\mu\text{g.}/100\text{ ml.}$ Thus, relation between the tocopherol level and the onset of deficiency symptoms was established. Zero levels were generally found in rats showing resorption sterility in all cases.

In man, a depletion experiment could not be carried out, since the manifestations of vitamin E deficiency were unknown.

Supplementation Experiment in Man. It was possible, however, to study the tocopherol content of the blood after extra-supplementation of vitamin E. In FIGURE 2 some results are given. The average tocopherol level was about 800 $\mu\text{g.}/100\text{ ml.}$ for eight persons who were given a daily dose of 15 mg. of tocopheryl acetate during a period of three weeks. The tocopherol level increased the the first week but in general it did not surpass 1200 $\mu\text{g.}/100\text{ ml.}$

Even when amounts of 120 mg. were fed (FIGURE 2, curve II) daily to a person with a tocopherol level of 1200 $\mu\text{g.}/100\text{ ml.}$, this was not further

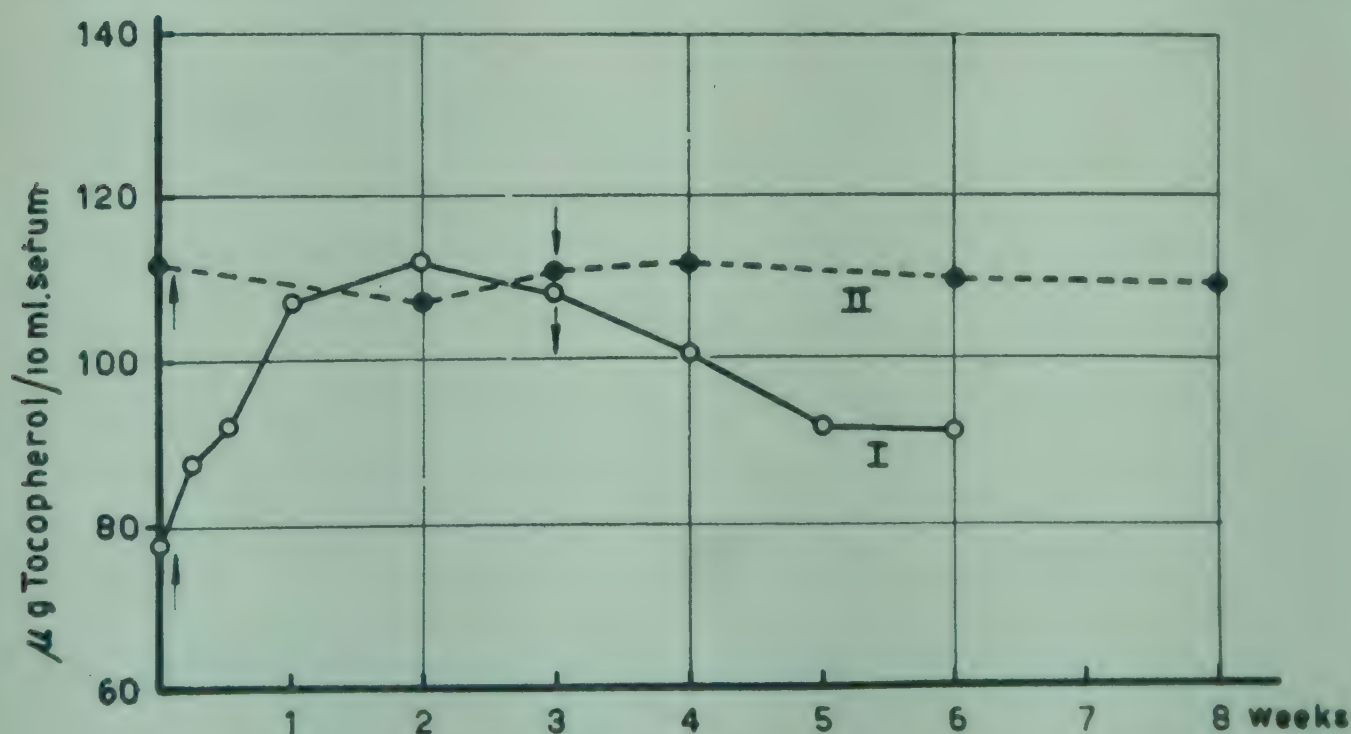


FIGURE 2.

I: 10 mg. Tocopherol acetate daily.
II: 120 mg. Tocopherol acetate daily.

increased. When tocopherol is administered as tocopheryl acetate, only free tocopherol could be demonstrated in the blood. Excretion of tocopherol in the urine was never observed.

These experiments indicate that when 15 mg. tocopherol is given daily, in addition to the 15 mg. present in the diet, the maximum blood level is reached in a few days. The maximum requirement is, therefore, about 30 mg./day but probably a little below this figure.

Therapeutical Investigations

In cooperation with Couperus¹ and d'Oliveyra,⁸ the therapeutic effect of tocopherol has been studied in neurological diseases such as muscular dystrophy and amyotrophic lateral sclerosis and in reproductive diseases such as sterility, habitual abortion, and hyperplastic uterus. In FIGURES 3, 4 and 5 the values of serum tocopherol are demonstrated in relation to

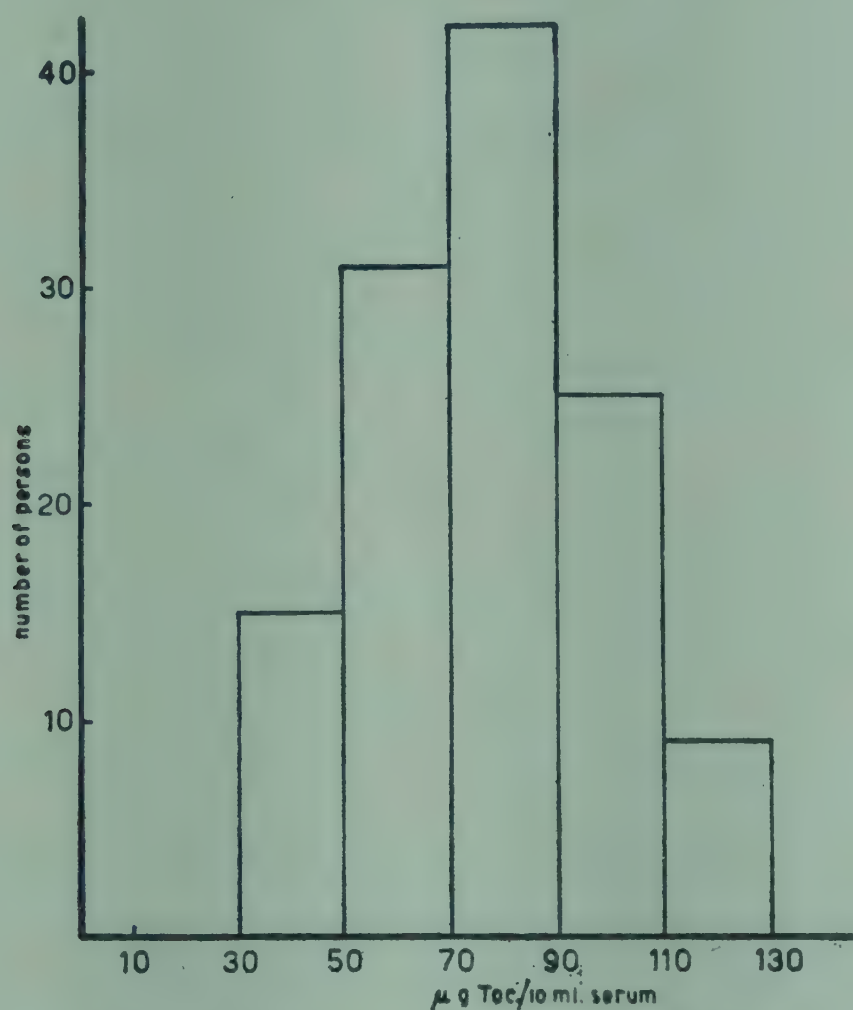


FIGURE 3. Normal persons; serum Tocopherol level.

frequency of 122 normal persons, 110 neurological patients, and 35 gynecological patients. The tocopherol level is not significantly reduced in the patients. When tocopherol was given *per os*, the blood level in patients increased just as in normal persons. Clinically, no improvement was observed in amyotrophic lateral sclerosis, multiple sclerosis, and muscular dystrophy.

These determinations in blood did not yield any concrete fact to support the view that vitamin E deficiency is a causal factor in the etiology of these diseases.

In some other diseases we found generally low vitamin E levels: sprue,

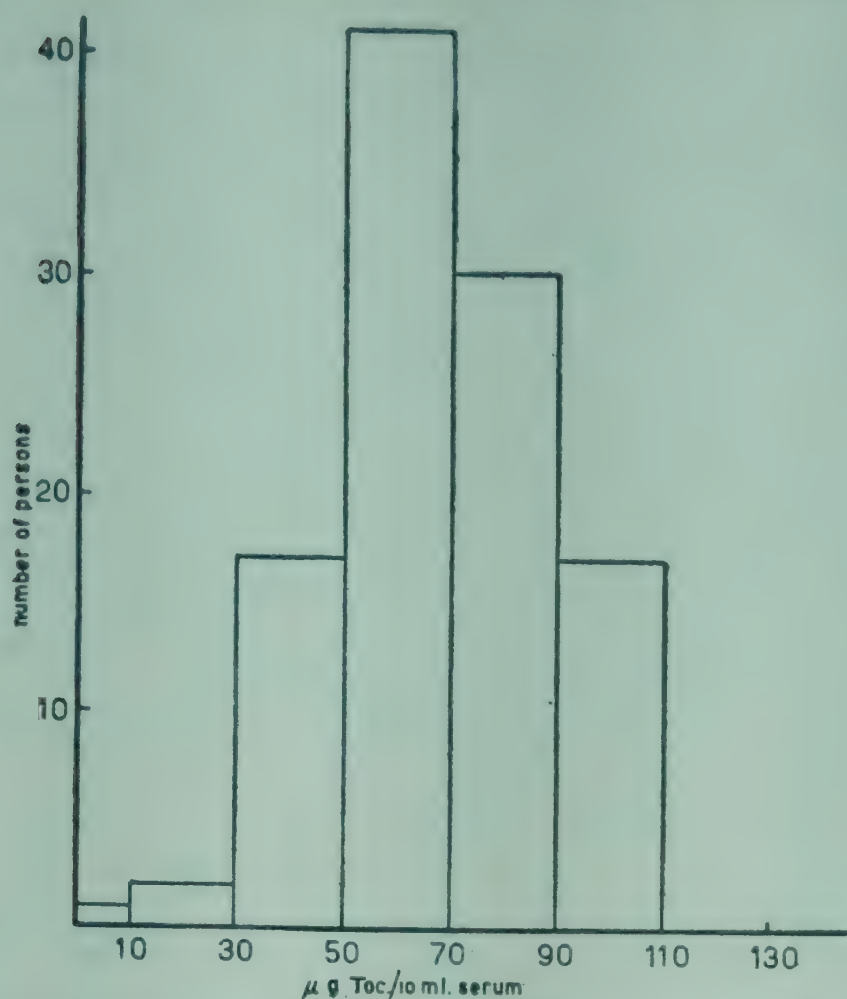


FIGURE 4. Patients with neurological diseases; serum Tocopherol level.

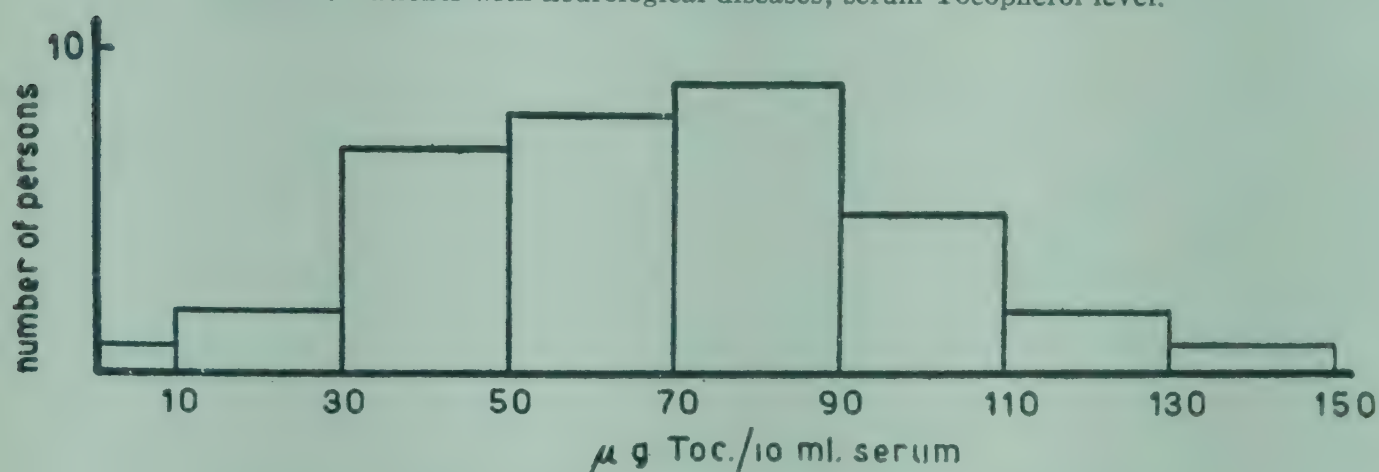


FIGURE 5. Patients with habitual abortion; serum Tocopherol level.

180 $\mu\text{g.}/100\text{ ml.}$; and opticus atrophy (Leber) and hyperplastic uterus, 600 $\mu\text{g.}/100\text{ ml.}$ In nephritis we noted hyper-tocopherolemia. In new born babies the average value was only 190 $\mu\text{g.}/100\text{ ml.}$ We expected to obtain from these studies any data concerning the minimum requirement for vitamin E in man.

The lack of correlation between serum level and clinical phenomena in these investigations does not rule out the possibility that, in the future, vitamin E deficiency in man will be traced.

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VITAMIN E IN FOODS AND TISSUES*

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Quantitative determinations of the amount and kind of tocopherols ingested and stored by different species of animals have revealed an interesting similarity and uniformity in the physiological utilization of vitamin E. Apparently, α -tocopherol is preferentially absorbed and deposited in tissues of chickens, cows, and humans. This strongly suggests that daily intakes and nutritional requirements of vitamin E should be expressed in terms of d, α -, rather than total, tocopherol.

In one experiment, laying hens were fed pure tocopherols at increasing levels as a supplement to a vitamin E-free diet. One object was to study the relation of level of tocopherol in the diet to its deposition in eggs. Another was to see whether evidence of *in vivo* conversion of non- α - to α -tocopherol would be found.

Pure, natural tocopherols, α -, γ -, or δ -, were given to hens by capsule in individual doses at levels of 100 up to 4,000 mg./week. The eggs were collected and tocopherol assays made on them for total and for γ - plus δ -tocopherols, α -tocopherol being estimated by difference.¹ When weekly supplements of tocopherol are plotted *vs.* tocopherol concentration in eggs (after 2 weeks on supplement), the latter appears to increase linearly with log dose fed. The hens on α -tocopherol supplement showed prompt and striking increase of tocopherol deposited in the eggs, whereas those given γ - or δ -tocopherol laid down proportionally much less tocopherol in eggs. For example, corresponding to the 400 mg. weekly tocopherol supplements, the respective concentrations in eggs were for α -, γ -, and δ -tocopherols—24.2, 5.7, and 2.3 mg./100 gms. fresh weight.

The relative efficiency of transfer of α -tocopherol to eggs by hens, as compared to γ - and δ -tocopherols, constitutes evidence of selective deposition in tissues of α - as compared to non- α -tocopherols by the animal body. Maximal values were calculated on a weekly basis. They were: α - 22.1 per cent, γ - 3.6 per cent, and δ - 2.0 per cent.

No evidence was found to show that there is *in vivo* conversion of non- α - to α -tocopherol. The δ -tocopherol-fed hen laid eggs with steadily increasing δ -tocopherol content as the level of supplementation was increased, while the γ -tocopherol-fed hen laid eggs which contained more than 90 per cent γ -tocopherol of total tocopherols at the higher levels of supplementation.

Increased deposition of tocopherol in cow's milk with increased supplementation was shown by a crossover type of experiment, wherein six cows each received six different levels of supplement. One supplement was a 60 per cent α -tocopherol preparation, and the other contained 90 per cent of γ - plus δ -tocopherols. Cows which received the larger supplements of α -tocopherol secreted much more tocopherol in the milk, while the non- α -supplements caused only slight increases. Milk from the former increased

* Communication No. 153.

in tocopherol content from 0.025 to 0.068 mg./gm. fat as the supplement was raised to 10 gm. total tocopherols daily. In the latter, the values were 0.022 to only 0.034 mg./gm. fat. This provides further confirmation of the selective deposition of α -, as compared to non- α -tocopherols by the animal body in its tissues and fluids.

Tocopherol values for all tissues of an adult male rat have been determined (TABLE 1). The rat received 1 mg. per day of α -tocopherol as a

TABLE 1
DISTRIBUTION OF TOCOPHEROL IN TISSUES OF AN ADULT MALE RAT*

<i>Tissue</i>	<i>Weight (gm.)</i>	<i>Tocopherol (mg./100 gm)</i>	<i>Concentration (mg./gm. fat)</i>	<i>Total tocopherol in the tissue (mg.)</i>
Blood cells	—	—	1.1	0.047
Suprarenals	0.04	34.0	0.7	0.014
Lungs	1.38	3.24	0.74	0.045
Spleen	0.58	5.1	1.1	0.030
Liver	10.6	2.52	0.51	0.269
Blood plasma	—	0.70	—	0.070
Gut	4.64	3.69	0.42	0.171
Kidneys	2.04	1.18	0.24	0.024
Thymus	0.49	1.7	0.7	0.008
Diaphragm	0.66	2.5	0.7	0.016
Heart	0.90	3.42	0.96	0.031
Penis	0.24	4.5	1.0	0.011
Seminal vesicles	0.72	2.6	0.7	0.019
Residue (skeleton Head, etc.)	91.0	3.55	0.57	3.23
Mesentery fat	5.85	6.0	0.071	0.349
Pelt	58.5	3.32	0.39	1.94
Muscle	86.0	1.33	0.54	1.15
Testes	3.18	2.26	1.05	0.072
Pancreas	1.62	5.48	0.20	0.089
Pituitary	0.01	90.	1.2	0.009
Central nervous system	2.49	1.62	0.16	0.040
Total				7.634

* Maintained on a vitamin E-free diet plus a supplement of 1 mg./day d, α -tocopherol.

supplement to an E-deficient diet. A total of 7.634 mg. was found in the rat. Pituitary and adrenal glands were very rich in tocopherol, having 90 and 34 mg./100 g., respectively. These high values for the single rat organ assays are confirmed by results found on pooled organs of other rats. Pituitary glands had 4 to 78 mg. tocopherols/100 g. and adrenals had from 26 to 332 mg. tocopherols/100 gm., fresh weight.

Of the remaining rat tissues, values on a fresh weight basis ranged from 5.85 mg./100 gm. for mesenteric fat to 0.70 mg./100 ml. for blood plasma. This concentration range is fairly close to that reported by Mason for tissues of rats maintained on low-E rations.² His range is 3.3 to 0.8 mg./100 gm. The total tocopherol in Mason's rat was estimated to be about 3 mg. in contrast to 7.6 mg. found here.

The tocopherol concentrations in rat tissues, per gram of fat, resemble

the values in human tissues, ranging from 0.1 to 1.1 mg. tocopherol/gram of fat.

Tissues from normal humans have been assayed for their total and γ -plus δ -tocopherol contents.³ Alpha-tocopherol was estimated by difference. For a man, a total of 3.4 gm. of tocopherols, of which 91 per cent was α -tocopherol, was calculated. The corresponding values for a woman were 8.1 gm. total tocopherols—88 per cent α -tocopherol. Human tissues showed a fifty-fold range of tocopherol concentration when expressed on a fresh-weight basis. The tocopherol concentration range, expressed on the basis of extracted lipid, was less than eight-fold: 0.2 to 1.2 mg./gm. in both man and woman. That tissues of the two human subjects seemed to have 90 per cent α - of total tocopherols indicates that α -tocopherol is preferentially stored in body tissues, since the usual daily intake of tocopherol consists of about $\frac{1}{2}$ α - and $\frac{1}{2}$ non- α - forms, according to dietary calculation.

Alpha-tocopherol is probably also absorbed preferentially to non- α -tocopherols in humans. Two studies from our laboratory indicate this. Absorption curves were obtained for 5 normal male adults who took 500 mg. doses of pure natural α - and of pure natural γ -tocopherol at random intervals. Values were determined on 0.06 ml. serum, obtained by finger-tip puncture.⁴ The absorption of α -tocopherol was increased over that of γ - in the 27-hour period measured (FIGURE 1). Blood from 18 normal subjects who had not

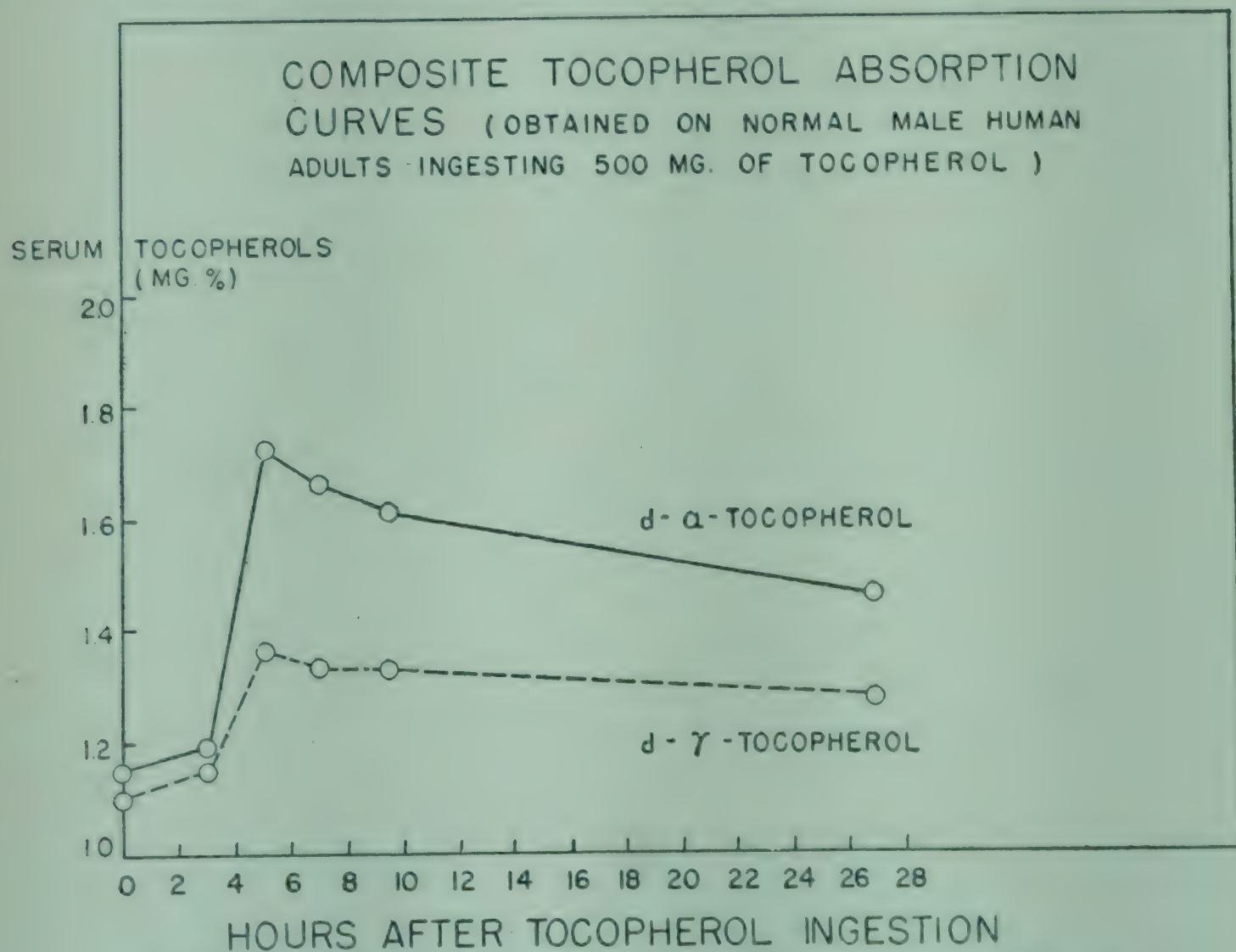


FIGURE 1. Comparative absorption of α - and γ -tocopherols by normal human adults.

received tocopherol supplements was assayed for total and for γ - plus δ -tocopherols. Total tocopherols averaged 1.00 mg. per cent for the series

and non- α -tocopherols only one-quarter of this. If α - and non- α -tocopherols occur in the diet in roughly equal amounts, this suggests that the former is more readily absorbed.

Estimates of dietary intake of vitamin E by humans throw some light on the probable human daily requirement. This should be expressed in terms of d, α -tocopherol or its biological equivalent, since the body seems to absorb and store it in the tissues with very much greater efficiency than it does the non- α -tocopherol forms.

With data on the total and non- α -tocopherol content of a variety of foodstuffs at hand, it has been possible to estimate the probable content of various diets in terms of α -tocopherol. Typical menus (3,000 Calories) which are adequate, as judged by current nutritional standards, have 10 to 25 mg. of α -tocopherol. The average daily per capita food consumption for the U. S. in a recent year, which yields a 3300 Calorie intake, provides 19 mg. of α -tocopherol.

Diets of a small series of women, who are subjects in a study of nutrition in pregnancy being conducted by Dr. N. S. Scrimshaw in Rochester, New York, have been evaluated for vitamin E. The α -tocopherol content ranges from 5.7 to 12.8 mg. per day.

In contrast, the α -tocopherol in numerous special diets, which are used for therapeutic purposes, amounts to less than 10 mg. (TABLE 2). Persons

TABLE 2
TOCOPHEROL CONTENT OF VARIOUS DIETS

Type diet	Total tocopherols (mg.)	Alpha tocopherol (mg.)
Low sodium,* 1,910 Cal.	9.32	7.22
Diabetic diet,† 1,600 Cal.	11.00	8.60
Reducing diet,‡ 1,000 Cal.	5.9 to 18.6	4.3 to 6.6
Fattening diet,‡ 3,000 Cal.	11.8 to 80.2	9.9 to 26.6

* Modern Medicine: 44. Feb. 5, 1949.

† WOHL, G. M. Dietotherapy, Clinical Applications of Modern Nutrition: 619. W. B. Saunders. Philadelphia and London. 1945.

‡ J. Amer. Med. Assoc. 139: 86. 1949.

subsisting on such diets for long periods of time might be expected to be in a poorer state of nutrition with respect to vitamin E than those who are very well fed.

The National Research Council has recommended a group of foods to be consumed daily which will supply adequate amounts of all recognized dietary essentials. The group of foods contains 5.7 mg. of α -tocopherol (TABLE 3), which is quite low as compared to the probable human daily requirement. It is doubtful that foods which would be eaten, in addition to these in the list, to round out caloric requirements would supply appreciably extra α -tocopherol unless a large amount of vegetable fat were included.

Loss of vitamin E in foods due to cooking would decrease the values just given. The amount of loss is variable. Deep-fat frying is very destructive. For example, losses of tocopherol in doughnut fat range from 70 to 90 per

TABLE 3

VITAMIN E CONTENT OF DIET RECOMMENDED BY NATIONAL RESEARCH COUNCIL*

<i>List 1</i>	<i>Calories</i>	<i>Tocopherol content</i>	
		<i>total (mg.)</i>	<i>alpha (mg.)</i>
Milk 1 pint	330	0.65	0.65
Egg, 1	80	1.00	0.60
Meat, Fish, or Fowl	225	1.10	0.95
Potato, 1 or more	200	0.14	0.14
Vegetables, 2 servings	75	1.00	0.50
Fruit 2 servings	150	0.50	0.50
Cereals and bread	500	3.20	2.40
Totals	1560	7.59	5.74

*National Research Council. Recommended dietary allowances: 16. Reprint and Circular Series. No. 122 1945.

cent. Potato chips contain 0.1 mg. tocopherol/gram of extracted fat, compared to the usual concentration of tocopherol in vegetable oils of about 1 mg./gm. Baking apparently destroys tocopherol to a lesser extent; extracted fat from a series of pies, cakes, and cookies had 0.26 to 0.76 mg. tocopherols/gram.

These considerations suggest that, under good dietary regimes, the human daily intake of d, α -tocopherol, derived from the diet, does not exceed 25 mg.

The experiments described here further indicate that there is selective deposition and perhaps absorption of α -tocopherol as compared to non- α -tocopherols for three species—cow, chicken, and man.

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Discussion of the Paper

DOCTOR K. HICKMAN: Referring to Dr. Quaife's data showing the analysis of a female human, I suggest that the results constitute an interesting discovery. That a man contains up to 5 grams and a healthy woman the enormous quantity of 5–10 grams of vitamin E (comparable with the quantity of cholesterol, 50 g.) is an exciting piece of information. One notes, too, that nine-tenths of the vitamin is in the adipose tissue and only one-tenth in what we may call the operating tissues. Dr. Quaife's analyses of food and Dr. Engel's measurements of efficiency of absorption show that the real daily intake is not more than 10 mg. The following conclusions, if not unescapable, seem to me to merit most careful examination.

- (1) For a woman, the dosage ratio is about 1000:1; the body contains a three years' supply of vitamin E.
- (2) The period of "half-adjustment" for the whole body is about two years.

- (3) The period of half-adjustment of the operative tissues could be as low as two months.
- (4) A short period of adjustment for the operative tissues would entail an even more extended period for the adipose deposits. This would parallel the clinical findings that certain E deficiencies respond rapidly while others resist years of treatment.
- (5) It may be assumed that there is free interchange between the adipose tocopherol and the active-tissue tocopherol. One would expect to find a definite partition coefficient established between them.
- (6) If the last point is true, a person putting on weight on a low E diet would dilute the tocopherol in the fat depots and this would actually extract vitamin E from the operating tissues. A woman putting on too much weight in pregnancy could rob the placenta and the fetus of α -tocopherol at the time that it is most urgently required. This might serve to explain the physicians' anxiety that the pregnant female shall curb her appetite.
- (7) A person (or animal) taking exercise after a period of rest will consume fat, thus making vitamin E available to the musculature just when needed. Perhaps Dr. Quaife's discovery gives us a glimpse of Nature's control mechanism for presenting α -tocopherol to the active tissues?

DOCTOR ADAMS: Infertile women in my medical practice become fertile when given tocopherol. Obese women known to be sterile often regain fertility following the loss of weight by systematic dietary restriction. Therefore, in essence, the experiment suggested by Dr. Hickman on the repartitioning of vitamin E has been carried out.

DOCTOR C. MACKENZIE (*Department of Biochemistry, Cornell University Medical College, New York, N. Y.*): Within what cellular component does Dr. Quaife consider vitamin E to reside?

DOCTOR M. QUAIFE: Our results on the distribution of tocopherol in tissues give no indication concerning the location of tocopherol in the tissues. The expression of vitamin E per unit weight of lipid, as well as per unit weight of fresh tissue, was made arbitrarily merely to afford easy means of comparison.

TOCOPHEROL CONTENT OF EDIBLE OILS SOLD IN THE MARKETS IN THE CITY OF BUENOS AIRES

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Introduction

According to an Institute publication in 1942 by Dr. Pedro Escudero¹ and collaborators, nutrient or edible vegetable oils are defined as "glycerides of fatty acids, of commercial standards of purity, obtained from various seeds or fruits, fluid at temperatures of 20°, of normal character and composition, with fatty acid content less than 1.5 per cent, calculated as oleic acid; of agreeable color and odor, free from rancidity, water, mineral oils, or chemical substances used in their purification."

(1) The consumption of vegetable oils is widespread in our country, representing, it is believed, over 60 per cent of the total fat utilized. This is in contrast to the habits of other countries, in which the major portion of fats consumed derives from animal sources.

(2) We have found no data in the literature as to the tocopherol content of these vegetable oils. It is of interest to supply this information, since the usual diet of our population is poor in tocopherols, especially in regions where the consumption of vegetables is very low. We have therefore analyzed 24 samples of vegetable oils purchased in the markets of the capital city.

Technical

We have employed the method of Emmerie and Engel,^{2,3} based on the reduction of ferric iron to ferrous by the tocopherols and the estimation of the ferrous ions by di-pyridyl. The carotenoids were removed by treatment with sulphuric acid, following the procedure of Parker and McFarlane,⁴ and the cholesterol, by adsorption in a column of "Florisil" (Floridin Co.). Slight modifications have been introduced into the method, as will be described. Recoveries have averaged around 90 per cent of the total tocopherol.

Necessary Reagents

(1) Sulphuric ether—washed twice with distilled water, distilled, and dried over anhydrous sodium sulphate.

(2) Benzene petroleum ether, 70–80 per cent—purified according to Parker and McFarlane,³ washed twice with concentrated H₂SO₄ (approximately 50 ml. to 500 ml. of ether), washed subsequently with dilute NaOH, finally with distilled water, and distilled.

(3) H₂SO₄—85 per cent (85 ml. of H₂SO₄ (D. 1.98) in 100 ml. of distilled water).

(4) KOH—2 per cent aqueous solution.

(5) Solution of ferric di-pyridyl—125 mg. of ferric chloride, 250 per cent alpha-di-pyridyl dissolved in 500 ml. of glacial acetic acid c.p. This reagent should be renewed every 15 days.

(6) "Florisil" (Floridin Co.)—purified as described by Emmerie and Engel.⁵ The "Florisil" is heated for 1 hour, with pure concentrated HCl, in a hot water bath, decanted, and the earthy sediment exposed to fresh HCl at room temperature for several hours, stirring from time to time. This is repeated 3 or 4 times, using fresh HCl. It is then washed with distilled water until the reaction becomes neutral, exposed to 3 changes of 95 per cent ethanol, and dried at room temperature.

(7) Fresh solution of 2N KOH in methanol.

Tocopherol Determination in Oils

The oil is saponified, taking into account the saponification index.⁶ One gram of oil and 2 ml. of 2N KOH solution in methanol are used, and the saponification is carried out at a temperature not exceeding 72° to 74°, in a hot water bath or over a micro-burner. The procedure should not last longer than 10 minutes. Then, 8 ml. of methanol and 10 ml. of distilled water are added and the mixture is extracted 3 times with 50 ml. of ether freed of peroxides. The ethanol extracts are combined, washed once with water, followed by 2 per cent aqueous KOH, and washed again with water, until reaction becomes neutral. The ether layer is filtered through anhydrous sodium sulphate and washed with peroxide free ether. The ether layer and the ether which has been used in washing the anhydrous sodium sulphate are collected and distilled at low pressure and room temperature in an atmosphere of nitrogen. The residue is dissolved in 15 ml. of petroleum ether, accurately measured, transferred to a graduated centrifuge tube with 3 ml. of sulphuric acid, stoppered, and inverted several times, until the aqueous layer is of a chestnut color as a result of the destruction of the carotenes and carotenoids. The supernatant should be colorless; if it is not, the sulphuric acid treatment must be repeated.

The ethereal solution is transferred as completely as possible to another

TABLE 1

<i>Material</i>	<i>Tocopherols (mg. per 100 gm.)</i>
Wheat germ oil	102.85
Cod-liver oil	Traces
Olive oil	Traces
Sunflower seed oil	75
Peanut oil	46.1
Cottonseed oil	94.4
Turnip seed oil	60.4
Grape seed oil 100%	1.9
Sunflower seed oil, edible	61.8
Sunflower seed oil 90, peanut oil 5, cottonseed oil 5	66.7
Sunflower seed oil 98.8, grape seed oil 1, palm oil 0.2	60.0
Sunflower seed oil 80, peanut oil 20	74.5
Sunflower seed oil 50, peanut oil 35, cottonseed oil 15	29.6
Sunflower seed oil 90, peanut oil 10	43.6
Sunflower seed oil 87, peanut oil 9, grape seed oil 4	60.1
Sunflower seed oil 85, peanut oil 15	76.6
Grape seed oil 90, olive oil 10	33.1

centrifuge tube, washed with 5 ml. of 2 per cent KOH, sealed, and centrifuged, avoiding evaporation. The petroleum ether is evacuated *in vacuo* in a current of nitrogen and the residue dissolved in 5 to 10 ml. of benzene. The benzene solution is poured through the column of adsorbent compound of "Florisil" and placed in an adsorption tube 80 x 12 mm. (before using the column, pure benzene should be poured through it once or twice to eliminate air). Then the tocopherol-containing benzene solution is passed through the column. The flask containing it is washed with benzene and also passed through the column, which is then washed 10 times with 5 ml. portions of benzene. The filtrates are collected and evaporated *in vacuo* to a volume containing 20 to 30 γ of tocopherols to 5 ml., in a graduated tube containing 10 ml. of a solution of di-pyridyl acetate.

The reading is made with a Pullfrich photometer with 30 mm. cell and filter S 50, after precisely 10 minutes. As a blank, a tube containing 10 ml. of the di-pyridyl reagent and 5 ml. of benzene is used. The readings are made from a curve based on pure solutions of tocopherol.

Results Obtained. The results are presented in TABLE 1. Each of the figures in the table is the average of four determinations from different samples of oils purchased in the markets in the Capital.

Remarks. From the data presented above, the following conclusions are drawn. Olive oil and grape oil contain practically no tocopherol. The remainder of the edible oils are a good source of vitamin E. Although the human requirement for vitamin E is not definitely known, 50 gm. of oil would supply approximately 20 mg., an amount above that usually given for therapeutic purposes.

Some oils contain more vitamin E than the amount stated by the manufacturers, which does not correspond to the true value.

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THE CHROMATOGRAPHIC SEPARATION OF THE TOCOPHEROLS

By A. Emmerie

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After the discovery of the methods for the chemical determination of the tocopherols, it soon appeared that the value found by these methods in various products did not always agree with values found by biological estimations. This is due to the fact that chemical methods of determination do not distinguish between the different tocopherols, which show very different biological activity. Alpha-tocopherol is the most active component. Beta-tocopherol has about 40 per cent of the activity of α -, whereas γ - and δ -tocopherol have little biological activity in the sterility-resorption test.

Tošić and Moore,¹ in their investigations on the α -tocopherol content of the unsaponifiable fractions of oils, used the chromatographic analysis with aluminum-oxide to remove interfering substances with little or no biological activity. In their experiments, α -tocopherol passed completely through the column but β -tocopherol passed only partly through (about 30 per cent).

In our experiments, we have tried to carry out a chromatographic separation between α -, β -, and γ -tocopherols. We used the natural forms of these tocopherols, which were kindly supplied by Dr. Robeson of Distillation Products Inc. All estimations of the tocopherols were carried out by our ferric chloride-dipyridyl method.

Experiments with Alumina. In our experiments, we used aluminum-oxide, British Drug Houses. Aluminum-oxide, Brockmann, was no improvement. The alumina was activated at a temperature of 106–108° C, and it was found that it must be used as soon as possible after activation.

With this adsorbent, a quantitative separation between α - and γ -tocopherol proved to be possible. We used a column 50 mm. in length and 13 mm. in diameter. The column was prepared by adding the adsorbent to the tube filled with the solvent: light petroleum with 1 per cent absolute ethanol (vol/vol). The tocopherols (1 to 1.5 mg.) were dissolved in the solvent (5 ml.). After passage through the column, the latter was washed with 21 ml. of solvent. Under these conditions, a separation of the α - and γ -tocopherols could be obtained. The separation by this procedure is possible only under carefully standardized conditions because, after elution of α -tocopherol, the γ -tocopherol very soon begins to pass through the column. This already occurs after the passage of about 2 ml. of solvent.

Under the same conditions, we have tried to separate α - from β -tocopherol. As β -tocopherol is less strongly adsorbed than γ -tocopherol, this separation offered many difficulties. We carried out experiments with various columns, using as solvents mixtures of light petroleum or benzene and ethanol, propanol, ethyl ether, and acetone. It turned out to be possible to separate nearly all the α -tocopherol from the β -tocopherol, but a quantitative separa-

tion could not be obtained. The limiting factor was the presence of traces of α -tocopherol in the filtrate when β -tocopherol began to pass through the column. This phenomenon is probably caused by the properties of the alumina.

Experiments with Floridin XS Earth. We used this earth for the separation of the tocopherols from interfering substances such as carotenoids and vitamin A. Using benzene as a solvent, these substances are adsorbed by the Floridin XS earth, whereas the tocopherols pass through the column. From the fact that the tocopherols are not adsorbed by this earth, it follows that it is a much weaker adsorbent than alumina. For the separation of the tocopherols with Floridin earth, only mixtures of light petroleum with benzene are suitable. In pure, light petroleum they were strongly adsorbed. It goes without saying that the earth must be standardized, otherwise the experiment is not reproducible. Some experiments on the separation of α - and γ -tocopherol with Floridin earth are given in TABLE 1.

TABLE 1

INFLUENCE OF THE RATIO LIGHT PETROLEUM:BENZENE (VOL/VOL) ON THE SEPARATION OF α - AND γ -TOCOPHEROL WITH FLORIDIN XS EARTH (5 ML. TOCOPHEROL SOLUTION; 1-1.5 MG. TOCOPHEROL)

Column mm.	Ratio	ml. eluate	% α	% γ
50 \times 9.6	2:1	15	73	5
"	"	25	100	56
"	4:1	25	74	0
"	"	35	99	10
"	5:1	35	70	0
"	"	45	92	0
"	"	55	96	3
70 \times 9.6	4:1	35	45	0
"	"	45	83	0
"	"	55	95	0
"	5:1	50	68	0
"	"	60	92	0
"	"	70	98	0

From this table it may be concluded that increase of the ratio, light petroleum: benzene, and of the length of the column results in a better separation of the two forms of tocopherol.

A separation between α - and β -tocopherol with Floridin XS earth is more difficult than the one between α - and γ -tocopherol. We used columns with a diameter of 13 mm. Taking a column of 60 x 13 mm. and a solvent mixture of light petroleum: benzene 5:1, a satisfactory separation may be achieved. After development of the column with 90 ml. of the solvent, the α -tocopherol passed the column; after 100 ml., traces of β -tocopherol appeared in the filtrate.

Separation of β - and γ -Tocopherol. A complete separation between these forms could not be reached. The following adsorbents were used: alumina,

zinc carbonate, calcium hydroxide, calcium citrate, calcium phosphate, and magnesium carbonate. With some adsorbents a partial separation was possible. In all cases, the γ -tocopherol was more strongly adsorbed than the β -form. In some cases, we observed a partial destruction of the γ -tocopherol.

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SERUM VITAMIN E LEVELS IN COMPLICATIONS OF PREGNANCY*

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Since the development of an accurate method for the estimation of total tocopherol in serum by Quaife *et al.*,^{1, 2} the direct biochemical investigation of the relation of tocopherol to complications of pregnancy has been practical. If sufficient differences exist between the vitamin E metabolism or supply in normal and abnormal pregnancy to be of etiological significance, it seemed possible that these differences would be reflected in the vitamin E blood levels. In this study, the serum tocopherol levels in abortion, prematurity, pre-eclampsia, and essential hypertension complicating pregnancy have been compared with those of normal pregnant women at corresponding stages of gestation.

Observations on the increase of vitamin E in the blood of pregnant women as gestation progresses are already available.^{3, 4, 5, 6, 7} Several European workers have also reported on the tocopherol content of the serum in women with spontaneous abortions, but the results are not consistent. Three of these^{7, 8, 9} fail to find any significant difference, while one¹⁰ reports significantly lower tocopherol values in abortion. In the paper by Rauramo in this monograph,¹¹ vitamin E values for "toxemia" are given which suggest that patients with this condition have lower blood serum levels. It is evident that these results are in part contradictory. Furthermore, the results reported by Rauramo¹¹ and several of the above are in a range of serum tocopherol encountered only rarely in Rochester, New York, patients. Also pertinent to the present study are the reports that there is no detectable variation in vitamin E blood levels before and after menstruation or at different stages in the menstrual cycle.^{6, 12}

D'Oliveyra¹³ does not find a lower vitamin E in pre-eclampsia and concludes that Shute's¹⁴ theory of the relation between pre-eclampsia and tocopherol is incorrect.

Methods

Plan of Procedure. Blood serum was taken from women at the time of threatened abortion or the development of pre-eclampsia. Similarly, serum was taken from apparently normal pregnant women at the time of one of their regular prenatal visits, usually the first, or at the time of their admission to the hospital at term. Many of these patients were in labor, but their tocopherol values did not seem to be affected by this. No attempt was made to secure these at any one time of day or in any fixed relation to meals. However, most of the samples were taken in the morning. Each sample was given a number and sent to the laboratory of Distillation Products Incorporated for macro-analysis.^{1, 2} The analyzing laboratory had no knowledge of the source of any sample. Similarly, the final diagnosis,

* Aided by a fellowship from the Nutrition Research Funds of Swift and Co., Chicago, Illinois. Assays were performed at Distillation Products Incorporated, Rochester, New York by Mr. H. A. Risley.
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established after the patient had actually aborted or delivered, was made before the results of the chemical determinations were known.

At first, only threatened aborters whose estimated blood loss was less than a normal period were sampled. Later, patients with any amount of bleeding were included in an effort to learn if blood loss could be responsible for any differences between abortions and controls.

Criteria for Diagnosis. Any patient with a previously normal blood pressure range, who showed a distinct rise of 20 mm. of mercury or more in diastolic pressure to a blood pressure of 140/90 or higher with or without albuminuria, edema, or other signs, was considered abnormal. If adequate evidence was at hand that this rise was of more than transient nature, *i.e.*, if despite bed-rest, sedation, *etc.* it persisted for several days in an out-patient or for several hours in a hospitalized patient, pre-eclampsia was diagnosed. If the pressure rose and remained above 150/100 for some period of time and if there was present a marked albuminuria, edema, and/or subjective symptoms of severe headache, visual disturbances, or epigastric pain, the condition was diagnosed as severe pre-eclampsia. Patients were considered to have hypertension antedating their pregnancy if (a) hypertension was present on previous admissions to the hospital, unassociated with a possible toxemia, or (b) hypertension was present at several successive clinic visits, the first of which occurred before the last trimester, and the hypertension persisted after delivery. Blood pressures consistently above 140/90 were considered evidence of hypertension. Abortions were diagnosed only when confirmed by pathological examination of tissue passed or curetted. Infected abortions and any suspected of being induced were excluded from this series.

Results

TABLE 1 demonstrates the progressive increase in serum tocopherol values with the progress of normal pregnancy. From the standard devia-

TABLE 1
VITAMIN E LEVELS IN NORMAL AND ABNORMAL PREGNANCY

<i>Weeks of gestation</i>	<i>Normal pregnancy</i>	<i>Abortions</i>	<i>Pre-eclampsia</i>
	<i>mg. %</i>	<i>mg. %</i>	<i>mg. %</i>
0-8	1.05 (.27)* N = 17	0.88 (.14)* N = 6	—
9-16	1.02 (.25) N = 36	0.96 (.24) N = 42	0.92 (.25)* N = 4
17-24	1.29 (.32) N = 36	1.05 (.28) N = 29	1.16 (.19) N = 4
25-32	1.38 (.30) N = 33	<i>Prematures</i> 1.46 (.39) N = 19	1.57 (.78) N = 10
33-40	1.51 (.44) N = 75	1.36 (.23) N = 6	1.65 (.50) N = 69

* Standard deviation in parenthesis

tion given in parentheses, it will be noted that there is a little more scatter with the values obtained for the women at term. Samples are reported from 197 women whose pregnancies were clinically normal. In the case of 25 women who delivered prematurely, it will be noted from TABLE 1 that the differences from the serum E levels of women delivering normally at term are small and variable. These differences have no statistical significance (TABLE 2).

TABLE 2
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs. Prematurity

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
25-32	51	0.80	.43
33-40	80	1.25	.21
25-40	2	X ² 2.05	.36

The values for pre-eclampsia are given in the third column of TABLE 1. Sixty-nine pre-eclamptic patients studied in the last trimester of pregnancy at the time of diagnosis of the condition showed gross values only slightly higher than the 75 controls for this period. It will be seen from TABLE 3

TABLE 3
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs. Pre-eclampsia

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
9-16	39	0.67	0.52
17-24	39	1.08	0.28
25-32	42	0.76	0.47
33-40	143	1.75	0.08
9-40	47	X ² 4.26	0.37

that the difference of 0.14 noted is not significant. Similarly, the differences to be found between women whose entire prenatal course was normal and women who eventually developed pre-eclampsia showed no significant difference in vitamin E, regardless of when they were sampled during pregnancy. These conclusions are further emphasized by reference to TABLE 4, in which

TABLE 4
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Mild pre-eclampsia vs. Severe pre-eclampsia

<i>Mean</i>	<i>σ</i>	<i>N</i>	<i>Mean</i>	<i>σ</i>	<i>N</i>	<i>t</i>	<i>P</i>
1.67	.43	44	1.61	.57	25	0.46	0.65

it will be noted that no significant differences in serum tocopherol values were found among severe pre-eclamptics, mild pre-eclamptics, and controls.

Seventy-seven women with spontaneous abortions were examined (TABLE 1). It will be seen that the mean for abortions occurring in the 17th to 24th week of pregnancy is nearly 20 per cent lower than that for normal pregnancies at this stage. Despite the variable bleeding encountered in these cases, the scatter of values is no greater than that found in the controls. Seven induced or therapeutic abortions, not shown in this table, were slightly lower in their tocopherol values than corresponding spontaneous abortions or corresponding normal pregnancies. Sixteen women with threatened abortions, who later delivered full-term babies, were found to have vitamin E values very slightly higher at the time of threatening than women whose pregnancy remained normal or who actually aborted. In each case the number of samples is too low for statistical treatment. However, the trend is directly contrary to that which would be expected if women who aborted spontaneously were actually lower in their serum vitamin E.

The statistical treatment of the data on spontaneous abortions is also presented in TABLE 5. It is necessary to make allowances in all comparisons

TABLE 5
STATISTICAL COMPARISONS FOR VITAMIN E SERUM LEVELS
Normal pregnancy vs. Abortion

<i>Weeks</i>	<i>D. of F.</i>	<i>t</i>	<i>P</i>
0-8	22	1.89	.08
9-16	77	1.00	.31
17-24	64	3.43	<.001
0-24	3	X ² 6.32	.10

of normals and abnormals for the increase in vitamin E with gestation. Accordingly, eight-week periods were selected as convenient intervals for comparison. Early in the collection of data, it was noted by sequential analysis that the differences in the 17-24 week group were of a greater magnitude than those in the 9-16 week group. Eventually, sufficient cases were assembled to yield the highly significant probability of less than 0.001, using the Student-*t* test for significance of differences between means. On the other hand, abortions in the 9-16 week group show essentially no difference, numerically or statistically, in comparison with normals for this same period. The few cases of abortions occurring spontaneously before 8 weeks can be added to the 9-16 week group for all practical purposes.

Since these data imply a difference before and after 17 weeks, and since the intervals were arbitrarily chosen, it is necessary to examine the effect of different groupings on this interpretation. When this is done, it appears that this change in serum E value relationships in abortions does occur in Rochester at approximately the 17th week of gestation. The small number

of cases occurring in any given week does not suffice to fix this point of difference exactly. When a Student-*t* test for difference of means is applied to the data for all spontaneous abortions occurring during the first 24 weeks of gestation, the probability of a significance of difference is very great. Such grouping, without consideration of differences within the group, would lead to the conclusion that all abortions, on the average, differ in vitamin E levels from normal pregnancies. However, such is not the conclusion properly drawn from our data, and the more rigid and properly applied chi-square test confirms this negative conclusion with a probability of only 0.10.

The differences observed cannot be accounted for by variations in blood loss. When the patients were sorted into groups, with bleeding exceeding that of a normal menstrual period before the sample was taken, equal to that of a normal menstrual period, or less, the differences observed in mean tocopherol values were not significant.

An effort was made to discover any existing differences in the serum of patients with essential hypertension. In 16 women with pre-existing essential hypertension, sampled during the last trimester of their pregnancy, values for serum E were only 0.12 mg. per cent higher than for normal women in this period. This gives a *t* value of 0.86 and a probability of 0.40. Similarly, comparison of values for the whole 29 essential hypertensives included in the study revealed no consistent difference in serum tocopherol.

If marked seasonal differences in vitamin E existed, many of the above comparisons could be invalidated unless these variations were taken into account. However, analysis of the data for seasonal variation in total tocopherol levels revealed only random changes, which appeared to be without significance.

Discussion

In both the data just reported and that of D'Oliveyra,¹³ no lowering of the serum tocopherol level can be detected in pre-eclampsia and eclampsia. This would seem to contradict the claim of Shute¹⁴ that "true" pre-eclampsia is associated with a deficiency of vitamin E. However, Rauramo,¹¹ in the paper presented in this monograph, reports 44 per cent of 32 women with a serum tocopherol level of 0.6 mg. per cent or below to show signs of toxemia, while only 8 per cent of 51 with serum tocopherol levels above 0.8 mg. per cent showed these symptoms. However, two cases of severe pre-eclampsia were among the 4 pre-eclamptics in the latter group. It would appear from the description of the methods employed (Rauramo⁶) that this difference in relative results is more likely to be due to differences in material than to differences in technique. We conclude from these data, therefore, that the correlation observed by Rauramo is not inherent in the pathogenesis of pre-eclampsia. Rather, the tendency of pre-eclampsia to occur in women in Finland with low vitamin E levels is probably the product of a secondary correlation of some sort and is a coincidental relationship as far as the pre-eclampsia is concerned. We are obtaining evidence from other vitamin studies in pre-eclampsia that secondary correlations of this sort have frequently confused the interpretation of the role of nutritional factors in this condition.

Having thus questioned the interpretation of apparently significant differences in vitamin E levels reported, by another investigator, for one of the complications of pregnancy, it is proper to inquire whether the same argument can be advanced against the differences reported here for abortions from 17–24 weeks. Such an objection is valid. However, unlike pre-eclampsia, where many differences between patients with this condition and normal pregnancy are known, there has been no previous clue to any consistent difference between abortions before and after 17 weeks. Thus, the recognition that the correlation found may be secondary rather than primary does not destroy its usefulness. In the case of abortions, the mere existence of a difference of any kind other than the temporal one between the earlier and the later abortions is of considerable theoretical interest. The present data suggest that there may be some difference in the etiological factors at work before and after 17 weeks, whether or not they have any direct relation to vitamin E. It is now important that the bases for the differences be sought. If the reason can be found through a search for secondary correlation, study of serum vitamin E will still have led us to a better understanding of human abortions.

However, it should be clear from the foregoing that the above data are most certainly not evidence that E therapy is of benefit in preventing abortions. In fact, our failure to find differences in serum vitamin E in women with abortions occurring during the weeks of gestation when vitamin E has been claimed to be therapeutically effective may be considered evidence against the value of E therapy in abortions. Likewise, the failure of many observers to find any efficacy of vitamin E in this condition is damaging evidence against any such hypothesis. Furthermore, when several investigators have found no differences in serum vitamin E levels in abortions occurring at any stage of pregnancy,^{7, 8, 9, 12} the relation of vitamin E to abortions would seem to be inconstant in general and perhaps coincidental in our data.

It is of interest to note that the series contained eight women with a history of two or more abortions and no living children, *i.e.*, presumptive habitual aborters.* These patients did not appear to differ from controls in their serum vitamin E. The failure of patients with essential hypertension complicating their pregnancy, whether with or without superimposed pre-eclampsia, to differ in their serum vitamin E values from normal controls has already been noted. This would seem to refute a suggestion of Shute¹⁴ that patients with essential hypertension in pregnancy should show a high vitamin E value in contradistinction to the low one which he predicted for “true” pre-eclampsia. It is further evidence that serum tocopherol values have no constant relationship to these two conditions. From the results cited, this conclusion would seem to apply also to premature labors.

Summary

No significant differences in the tocopherol serum levels of 69 patients with pre-eclampsia, 16 with essential hypertension, or 25 with premature

* Only four in this group met the definition for habitual abortion (*i.e.*, three or more consecutive abortions); hence, the more liberal criterion was used.

labor could be detected in comparison with 197 normal pregnant women at a corresponding stage of pregnancy. The increase of serum vitamin E with gestation was confirmed. Forty-two abortions, 9-16 weeks, averaged 0.96 as compared with 36 normals averaging 1.02 mg. per cent. The difference has no statistical significance. The serum of patients with abortion during the 17th to 24th weeks of pregnancy did differ significantly from corresponding controls. The mean value was 1.05 mg. per cent for the 29 abortions and 1.29 for the 36 normals, with a probability of significant difference by Student-*t* test of less than .001. The earlier and later abortions do differ from each other in this study in the relation of their mean serum values to those of normal pregnant women. There is no evidence that the difference observed is a primary one or related in any way to the etiology or pathogenesis or therapy of abortion.

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Discussion of the Paper

DOCTOR P. GYÖRGY (*School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*): It appears to be highly questionable whether, in complications of pregnancy, serum vitamin E levels in themselves or any phase of the vitamin E metabolism of the mother may sufficiently characterize the condition in question. The transfer of vitamin E through the placenta to the fetus and the vitamin E content of, and its distribution in, the fetus seem to have received only scant attention in the relevant literature. Pertinent data may shed more light on the rôle of vitamin E in complications of pregnancy.

DOCTOR E. SHUTE (*The Shute Institute, London, Ontario, Canada*): This work tends to explain two old claims of mine. The differential between abortions and miscarriages demonstrated by Dr. Scrimshaw is reflected in the observation reported some years ago¹ that vitamin E salvages 72 per cent of abortions and prematures but fully 85 per cent of miscarriages. As early as the first vitamin E symposium, I had mentioned my inability to discover any value in vitamin E for women who habitually aborted. The only rôle that vitamin E has in this last condition is in improving the *quality* of the semen before conception occurs.² Further, I would like to repeat an old objection to women being labeled "habitual aborters" before

they have experienced at least three consecutive premature interruptions of pregnancy.

One wearies of hearing all the late toxemias lumped together as pre-eclampsics. The facts that so many treatments for this condition have been tried in the history of obstetrics and that all have "prevented" the onset of convulsions in the great majority of cases merely emphasize the view that few of these patients are really pre-eclamptic. Most of them could not possibly convulse. Instead, they tend to terminate in placental detachment, foetal death, and/or evidences of renal-vascular disease. Such a crude approach as the single classification of "pre-eclampsia" tends to vitiate the whole literature on the subject, and therefore any conclusions drawn from such a study are open to serious challenge.

My own views on the late toxemias are identical with those I expressed at the first vitamin E symposium. I was misquoted by the present authors when they said I held that pre-eclampsia was a low-E phenomenon. I have always held and published an opinion exactly the reverse; namely, that it was intimately associated with estrogen deficiency. Indeed, I seem to have initiated the estrogen therapy of pre-eclampsia and eclampsia,^{3, 4} and have used virtually nothing else for the past 12 years. I have also reported, and soon will publish more evidence for, the belief that vitamin E actually is contra-indicated in pre-eclampsia and can even initiate convulsions in severe cases.^{5, 6}

It would be somewhat illogical to think that tocopherol therapy had a place in the management of miscarriage but not in the management of such similar conditions as abortion and prematurity. I feel that alpha-tocopherol has a great role in both the latter as well, and should stress the value of *preventing* premature births rather than *treating* prematures once they are delivered. The latter procedure is relatively sterile, leaving a vital problem in suspension. We have shown that in Canada during the last war there would have been no population loss had the prematures been saved.⁷ Yet we mobilized hundreds of doctors to salvage the casualties of war and can find almost no one interested in the *prevention* of prematurity! It is a truly astounding hiatus in medical thought!

Finally, the authors demonstrate, if they show anything, that E-therapy in miscarriage and allied conditions is aimed not at a pre-existing E deficiency in the body, but at something much different. The close parallel between the results of vitamin E therapy in these cases and those achieved by progesterone would suggest that both work on a common denominator, namely, an excess of estrogen in the maternal organism, and that is exactly what I believe.^{8, 9}

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DOCTOR N. SCRIMSHAW: I am afraid that Dr. Shute has misunderstood the conclusions of our paper. We have reported only the results of tocopherol blood serum level determinations in the conditions discussed and have attempted to do so in a completely objective manner. Others may interpret the results differently. However, we have tried to point that the absence of any demonstrable serum vitamin E deficiency in early abortions or cases of prematurity seems to us to make it unlikely that vitamin E therapy is required or of value in these conditions. We have also stated our reasons for believing that the slightly lower tocopherol findings reported in our series for late abortions do not reflect any primary etiological relationship to vitamin E. For these reasons, we do not believe that our results can explain a 72-85 per cent salvage rate from vitamin E therapy, a claim which seems to us most unlikely because of the wide recognition of multiple causative factors in abortions and prematures (*i.e.*, "blighted ova," congenital anomalies, endocrine dysfunction, hypertension, nephritis, pre-eclampsia, psychological factors, general nutritional status, etc.). Furthermore, claims for the effectiveness of vitamin E therapy cannot be based on the assumption that threatened abortions or prematures subsiding on treatment with vitamin E necessarily do so because of its administration, especially in the absence of controls. Anyone who believes strongly in the value of vitamin E therapy is most sincerely urged to demonstrate its values by means of an adequately controlled series with carefully defined diagnostic category. As far as we have been able to determine, this has never been done.

It should be very clear from our paper that we have not attempted any therapy with vitamin E and do not recognize any indications for such therapy in the diseases discussed. In our discussion we referred only to *presumptive* habitual aborters, because most of these women had only two abortions and not the three consecutive ones necessary for the diagnosis. The results were still considered to be of interest.

Dr. Shute suggests that only a small group of the patients which we define as pre-eclamptics are actually true pre-eclamptics, manifesting a disease identical in pathogenesis to eclampsia. In contrast, we agree with the vast majority of obstetricians that, when cases of nephritis and essential hypertension are carefully excluded, the remaining group with elevated blood pressures and albuminuria represent a single pathological process whether or not they convulse. Objective evidence supporting this from our laboratory includes the homogeneity of electrophoretic patterns (Federation Abstr. **8**: 368. 1949), similarity in most clinical manifestations (Amer. Jour. Obst. & Gyn. **54**: 3-19. 1947), and the distribution of many biochemical findings (unpublished data).

In a seminar discussion in Rochester in the fall of 1946, Dr. Shute clearly stated that, although he had never done blood level determinations of tocopherol, he would predict a high vitamin E blood level (associated with estrogen deficiency) in the cases he defined as pre-eclamptics. He stated that the majority of so-called toxemias were not to be considered as true

pre-eclamptics by his standards and might be expected to have a low vitamin E blood level. The possibilities for direct experimental testing of this hypothesis intrigued us and stimulated the present report. The significance of our findings in regard to pre-eclampsia is that we find no tendency for the tocopherol level to be affected in any way by this condition, regardless of its clinical severity or exact nature.

THE SIGNIFICANCE OF SERUM-TOCOPHEROL LEVELS DURING PREGNANCY

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To find out the vitamin E balance in the organism is still a difficult task, liable to different interpretations. It is not possible to perform vitamin E tolerance tests by measuring the excretion of this substance after administration of large doses. To determine vitamin E in the serum there are several more or less unspecific methods¹ which have been employed with a view to obtaining some kind of idea regarding the vitamin E balance in the organism. Yet the vitamin content of the serum is not in itself a particularly good criterion for assessing the vitamin balance of the organism, the blood being, in the first instance, a means of conveyance, and therefore the determinations made from it do not provide any evidence regarding the vitamin quantities which may be stored in other tissues.

The knowledge we possess on the metabolism of vitamin E in the human organism during pregnancy is derived chiefly from determinations of tocopherol in the serum. Rauramo^{1, 2} has demonstrated that the tocopherol content of the serum increases during pregnancy. Varangot³ and Straumfjord and Quaife⁴ have come to the same conclusion. It can, therefore, be considered a fact, although d'Oliveyra⁵ was unable to find in his series any difference in tocopherol contents of the serum of pregnant and nonpregnant women. On the other hand, the amount of tocopherol in fetal blood is exceedingly low, as shown consistently by d'Oliveyra, Varangot, and Straumfjord and Quaife.

According to Varangot, vitamin E passes through the placenta, since a significant amount of tocopherol administered to the mother somewhat increases the tocopherol content in the fetal serum. It is apparent, therefore, that the fetus gets its share of the higher than normal tocopherol level in the maternal serum, but evidently loses it in its own metabolism. Rauramo and Somersalo⁶ have shown that even small premature babies are able to absorb tocopherol by mouth, and Rauramo¹ has observed that the tocopherol content of the maternal blood rapidly diminishes during the period of nursing. Moreover, Rauramo¹ has found that, at least in wartime under unfavourable nutritive conditions, the tocopherol content of the maternal serum drops below the normal level in consequence of a prolonged and ample nursing. Faaborg-Andersen⁷ finds no difference between the serum tocopherol content of abortion patients and normal pregnant women.

Shute⁸ considers the vitamin E medication effective in habitual interruption of pregnancy during 16–28 weeks. He regards vitamin E as a potent anti-estrogen and determines the vitamin E balance in the body by means of the blood estrogen test. Shute⁹ has also obtained good results in the treatment of toxemia patients with vitamin E, if the estrogen content of their blood was high. According to him, such toxemia patients do not have eclampsia at all, irrespective of the treatment administered to them, eclamp-

sia manifesting itself only in those patients who have a low estrogen content of the blood and where, consequently, the vitamin E medication is of no avail. D'Oliveyra⁵ had tested the theory formerly advanced by Shute, regarding the correlation of toxemia and vitamin E contents, by determining these contents in the serum of eclampsia patients, and he considers, on the basis of the high values obtained by him, that Shute is wrong.

In my endeavours to find out whether the low tocopherol content in the serum has an unfavourable effect on the course of pregnancy, I have performed determinations of the serum tocopherol content during pregnancy in about 200 expectant mothers at the Maternity Centre of the Women's Clinic of the University, Helsinki. For the determinations, the modification of Emmerie and Engel's ferric chloride-dipyridyl reaction described by Rauramo¹ was used. After the delivery, follow-up examinations were made on the course of the pregnancy and the delivery and their complications. The history of the patient's previous pregnancies was studied simultaneously. Up to the present moment, follow-ups have been performed in 136 cases. The cases are grouped in accordance with the classification described by Rauramo¹ based on the tocopherol content of the serum, using 0.6 and 0.8 mg. per cent as threshold values. TABLE 1 illustrates the results obtained.

TABLE 1
RELATIONS OF THE SERUM TOCOPHEROL CONTENT TO SOME COMPLICATIONS OF PREGNANCY

Tocopherol contents in the serum (%)	<0.6 mg.	0.6-0.8 mg.	>0.8 mg.	Total
Symptoms of toxemia (%)	44	21	8	22
History of abortions or premature births (%)	41	34	14	28
Hyperemesis (%)	19	21	14	18
Number of cases	32	53	51	136

When studying the tabulated values, one should note particularly the distribution of toxemia symptoms among the different groups. Symptoms of toxemia appeared in 22 per cent of the whole material, which is a somewhat higher figure than what was simultaneously ascertained in the maternity wards of the Women's Clinic, where the percentage was about 10 per cent according to the annual report. This was due, principally, to three factors: (1) patients who are expected to develop complications are sent to the Clinic's Maternity Centre from elsewhere; (2) some of the symptoms observed at the Centre were cleared up by treatment before the delivery; and (3) the material had been collected mainly in early spring, at which time the incidence of toxemia in Finland is at its height (as demonstrated by Schroderus (Rauramo)¹⁰).

As to the distribution of toxemia symptoms into different groups, the one below 0.6 mg. per cent had considerably more patients revealing symptoms of toxemia than any other group. In spite of the scarcity of the material, we can observe that those patients, whose tocopherol content in the serum during pregnancy is below 0.6 mg. per cent, are evidently more liable to toxemia than patients with a tocopherol content in the serum of over 0.8 mg. per cent (difference 36 ± 9.8 per cent).

It is noteworthy, however, that the only two cases of severe pre-eclampsia in the series were in the group surpassing 0.8 mg. per cent. Yet the results obtained by me have induced me to investigate the cause of this correlation between the serum tocopherol contents and the manifestation of toxemia symptoms, and whether it is possible to reduce the incidence of toxemia by tocopherol therapy, as assumed by Shute.⁹ In Finland at least, it does not seem to be a question of merely a tocopherol deficiency in the diet, but rather of some disturbance of the vitamin E metabolism in the organism, and, according to my material, this disturbance is already in existence before the manifestation of the toxemia symptoms, evidently not being directly produced by toxemia. The material I have collected on the effect of tocopherol therapy on toxemia is as yet too small to permit any definite conclusions to be drawn.

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THE SHUTE TEST FOR CHECKING UNBALANCE PRODUCED BY LACK OF VITAMIN E

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Evan Shute has demonstrated¹⁻⁴ the presence of an anti-proteolytic factor in the blood serum of aborting women. Such sera, under the action of trypsin, reveal no freeing of acid radicals. Vitamin E, under certain circumstances, corrects this anomaly. In 120 pregnant women studied in the Sao Paulo Maternity Hospital, we have recorded 53 who presented tryptic digestion resistance; that is, they presented positive reactions. The following types of curves (summarized in FIGURES 1 and 2) were obtained by us:

(A) Curve representing negative Shute test obtained with pregnant women under good nutritional conditions. No resistance to tryptic digestion; Wassermann positive; normal evolution of pregnancy.

(B) Curve representing an initial resistance period, with unstable vitaminic balance. Digestion proceeded slowly, with some resistance. Pregnancy developed under precarious conditions. There seemed to exist some vitaminic unbalance due to defective nutrition. Delivery at term.

(C) Positive curve showing complete resistance to digestion indicating lack or unbalance of vitamin E. Precarious condition of nutrition; negative Wassermann, patient poorly nourished, having children with low resistance and premature deaths.

(D) Curve modified by means of vitamin E administration. Patient showed a poor obstetric history, with positive Wassermann and positive Shute test (curve D1). An anti-luetic treatment and use of vitamin E were prescribed. The latter treatment was stopped by patient after third application, but the anti-luetic treatment continued. There was threatening of abortion, after which the patient visited the medical services. Vitamin E was recommended again. After the sixth injection of vitamin E, a new Shute test was carried out, with negative results (curve D2). Pregnancy continued normally.

A positive Shute reaction in one patient (V.R.) was changed to negative by vitamin E administration, then reversed to positive by stopping vitamin E treatment, then again changed to negative by giving vitamin E.

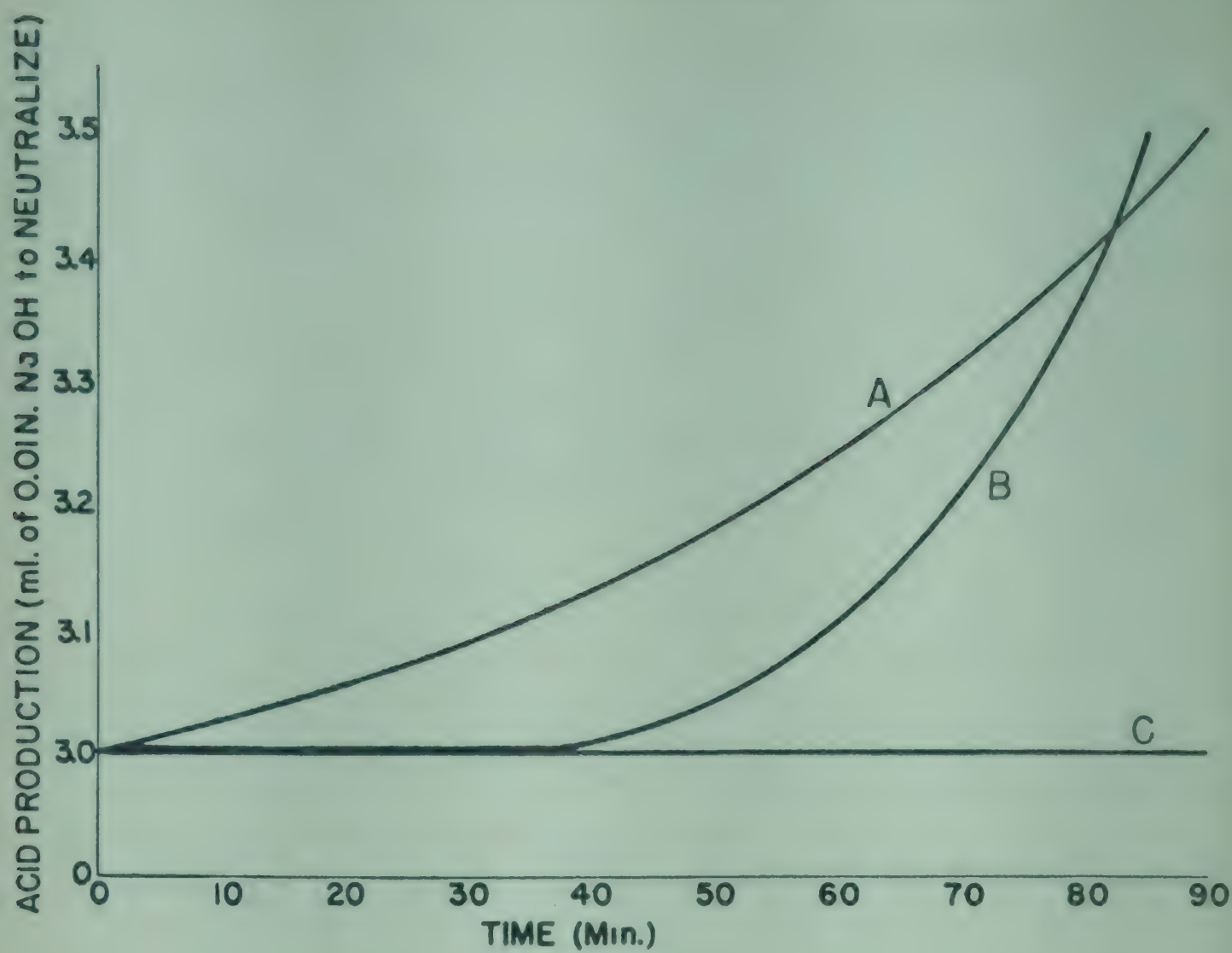


FIGURE 1.

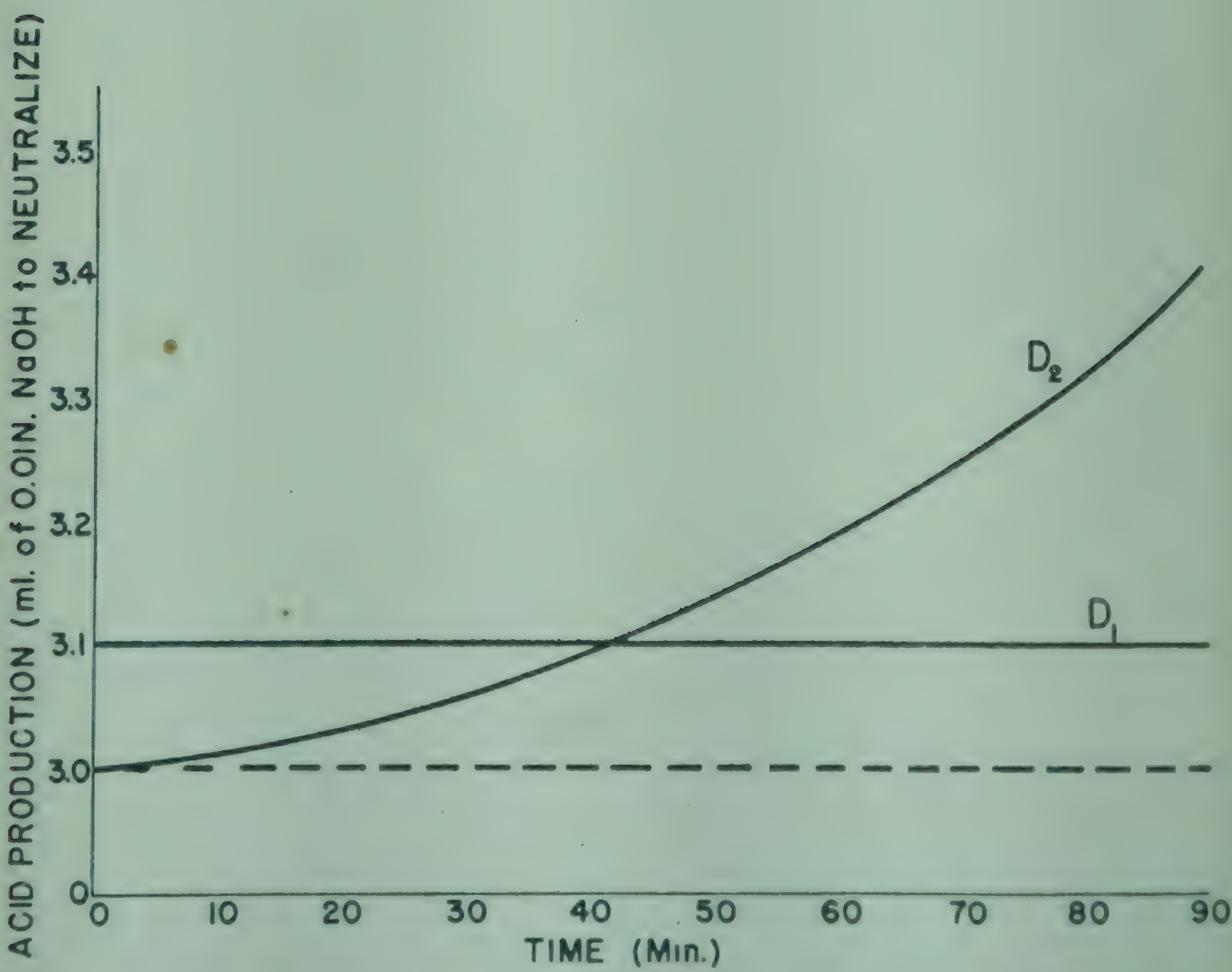


FIGURE 2.

Conclusions. (1) The Shute test can supply useful data in cases of unbalance of vitamin E. (2) Taking into account the occurrence of abortions in cases of vitamin E unbalance, the Shute test can help clinics. (3) This is not specific, but a guidance test.

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PLASMA TOCOPHEROLS IN HEALTH AND DISEASE*

By William J. Darby, Mary Ellen Ferguson, Robert H. Furman, Janet M. Lemley, Con T. Ball, and George R. Meneely

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Efforts to interpret the significance of altered concentrations of vitamins in blood plasma or serum must be preceded by descriptive studies of factors associated with variations in concentration. Numerous recent reports of tocopherol levels of healthy individuals have appeared.^{2, 5, 7, 9} These reports are in agreement with the finding that plasma or serum total tocopherol concentration in healthy adults clusters around the value of 1.0 mg. per 100 ml. Several important variations in vitamin E levels have been recorded: Straumfjord and Quaife⁸ showed that plasma vitamin E concentrations rise with the progress of pregnancy to a level approximately 65 per cent higher at term than for non-pregnant women. Similar results have been reported from this laboratory.¹ The vitamin E level in the plasma of newborns is low, with a mean of $0.34 \pm .12$ mg. per 100 ml.⁸ Lower-than-usual tocopherol levels have been reported in malnourished patients,³ in celiac disease,⁵ and in sprue.² Normal to slightly lower than usual values have been encountered in certain of the myopathies.^{9, 10} Pronounced deviation from healthy levels has not been found in patients with diabetes¹¹ or heart disease.⁴

The purpose of the present report is to summarize some recent observations on plasma tocopherol levels in healthy subjects and patients.

All of the determinations have been made on oxalated plasma from venous blood. The convenient method for total tocopherols developed by Quaife and Harris⁷ and simplified by Quaife and Biehler⁶ has been used throughout.

Studies reported in detail elsewhere⁴ have indicated that plasma tocopherol levels in a series of healthy laboratory workers were distributed over a narrow range and that the mean plasma tocopherol concentration was somewhat higher than occurred in a series of randomly chosen medical patients or patients with heart disease. On the other hand, similar distributions were observed between patients with various medical diseases and patients with heart disease. A slight but significantly positive correlation was found between plasma tocopherol level and age of the patients. The healthy controls in this series did not embrace sufficient age-span to test for the presence of this correlation in the non-patient group. This study, together with some of the reports just cited, permits the conclusion that medical patients admitted to low-cost hospital service exhibit greater variability of tocopherol concentrations and somewhat lower mean values than do healthy young middle-class laboratory workers. In addition, there is a tendency for higher tocopherol concentrations to occur in the older patient-group than in the younger.

In order to study further factors influencing plasma tocopherol values in

* These studies were supported by grants from Distillation Products, Inc., the National Vitamin Foundation, the U. S. Public Health Service, the Eli Lilly Company, and the Ciba Pharmaceutical Products, Inc.

healthy women, determinations were made at three-day intervals on seven subjects throughout 12 menstrual cycles. The results are summarized in FIGURE 1 and indicate that no constant pattern of variation occurred.

VARIATION IN PLASMA TOCOPHEROL DURING MENSTRUAL CYCLE
(7 SUBJECTS, 12 CYCLES)

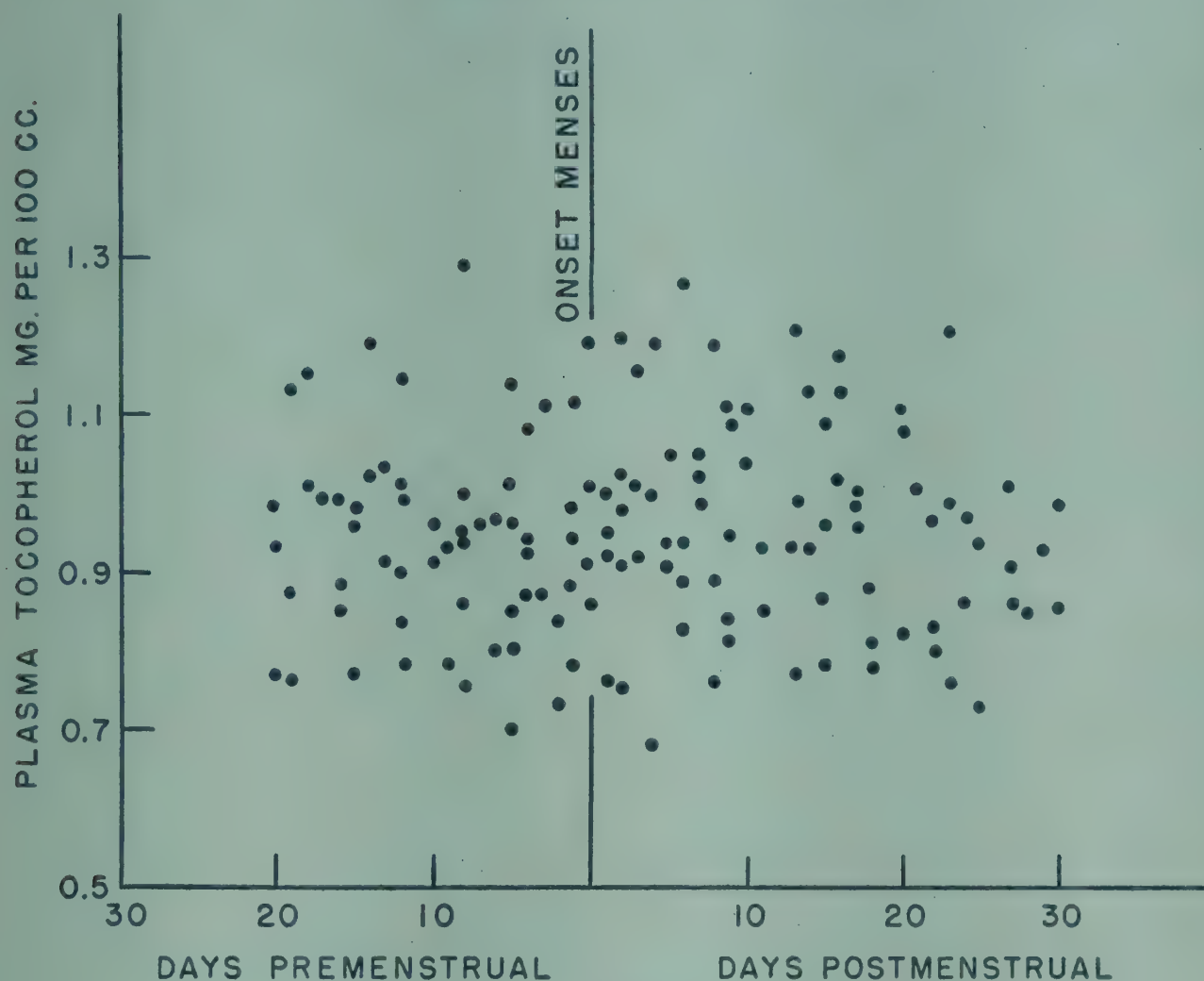


FIGURE 1.

As a part of the Vanderbilt Cooperative Study of Maternal and Infant Nutrition, vitamin E levels are being determined on approximately 2,000 pregnant women. The data have not all been assembled to date, but a preliminary analysis¹ of a sample of the group has indicated that mean values for successive trimesters of pregnancy were 0.84 ± 0.03 , 1.07 ± 0.01 , and 1.24 ± 0.01 ; and, at the six week post-partum examination, they were 0.96 ± 0.04 mg. per 100 ml. It is of considerable interest that the percentage change in total tocopherol concentration from trimester to trimester of pregnancy parallels very closely the percentage change in serum carotene. It is of further interest that the percentage increase in both of these substances appears to be greater than the percentage increase in cholesterol or total lipids during pregnancy. We shall ascertain whether correlation exists between different tocopherol levels and the clinical course of pregnancy and report these at a later date.

We have surveyed the tocopherol concentration of plasma in 200 patients with a variety of medical diseases. All of these were patients in the Vanderbilt University Hospital. The diseases included a variety of gastrointestinal abnormalities, hematologic and nutritional disorders, endocrine dysfunction,

infectious diseases, malignancies, psychiatric disorders, and degenerative diseases (such as arthritis, cardiovascular disorders, nephritis, diabetes, and a few other miscellaneous conditions).

A classification of diagnoses in order of increasing plasma vitamin E concentrations immediately revealed an interesting pattern, which has been generalized in FIGURE 2. This figure depicts the range of tocopherol values

SPECTRUM OF PLASMA TOCOPHEROL

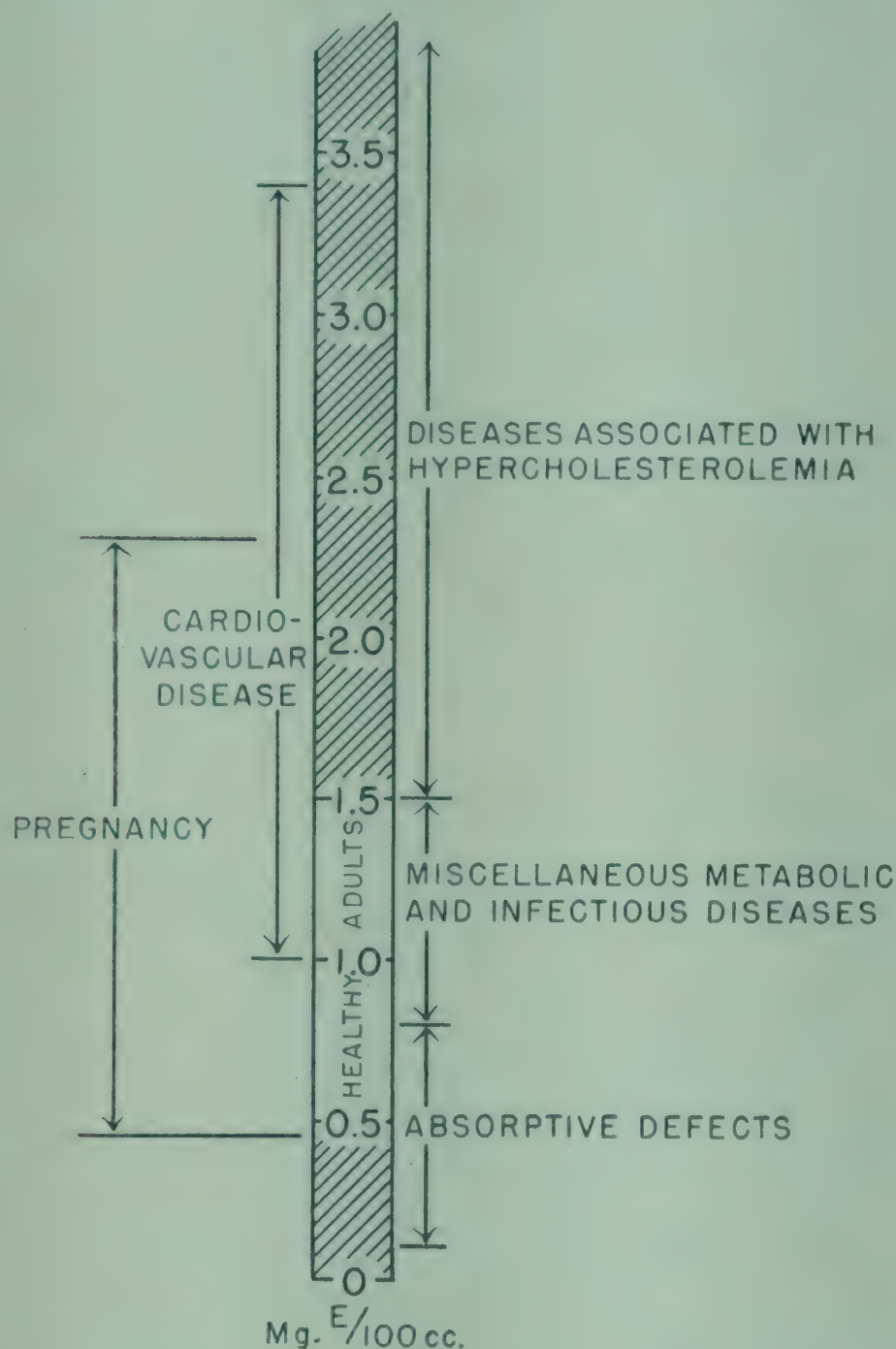


FIGURE 2.

encountered in certain broadly classified diseases. It is to be noted that healthy adults fall within the range of 0.5 to 1.5 mg. per 100 cc. Values encountered during pregnancy span the healthy range, with extensions into higher levels. Patients with cardiovascular disease tend to fall into the upper ranges associated with health and to extend considerably above this range. Patients with miscellaneous metabolic and infectious diseases seem to exhibit levels no different from those encountered in healthy adults. These diseases include such conditions as ulcerative colitis, hemolytic anemia, multiple sclerosis, vulval leukoplakia, hypertrophic arthritis, mesenteric

lymphadenitis, hypochromic anemia, ichthyosis, poliomyelitis, acromegaly, carcinoma of the mouth, anxiety states, infectious hepatitis, diarrheas other than steatorrhea, pernicious anemia, surgical castrate with menopausal syndrome, tuberculosis, post-menopausal osteoporosis, non-thrombocytopenic purpura due to unknown cause, macrocytic anemia of pregnancy, and so forth. Some diseases associated with hypercholesterolemia appear to be associated with higher-than-usual tocopherol levels. These cases include such conditions as carotenemia with unexplained hypercholesterolemia, carotenemia associated with diabetes and nephrosclerosis, xanthomatosis with hypercholesterolemia, cerebral thrombosis, intercapillary glomerulosclerosis, and, in one case, pre-eclampsia.

This association of hypercholesterolemia and hypertocopherolemia is not due to an effect of cholesterol on the chemical determination of tocopherol. It may, of course, merely be a reflection of the increased lipid-carrying power of the serum in these various abnormal states. In this connection, it is of interest that pregnancy and cardiovascular disease are both often associated with increased blood lipids. Conversely, those absorptive defects found to be associated with unusually low tocopherol concentrations are, also, often noted to exhibit low cholesterol values.

The absorptive defects encountered in the low-tocopherol group include sprue, fibrocystic disease of the pancreas, biliary obstruction, Whipple's disease, and diarrhea associated with achlorhydria. Two explanations of low vitamin E values in such states occur to one: (1) lipid-soluble vitamin E is poorly absorbed; and (2) due to some metabolic disturbance, the lipid-carrying power of the blood is reduced. While it is impossible to decide categorically between these two possibilities, the widely recognized association of these disorders with defects in gastrointestinal absorption would indicate that such a derangement reduces the amount of tocopherol available from the diet and thereby results in a decrease in blood levels of this vitamin.

We have also noted low values in patients with nutritional macrocytic anemia, pellagra, carcinoma of the stomach, and glossitis and cheilosis (of nutritional origin?). This is in keeping with the findings of Harris, *et al.*³

It would appear, inasmuch as patients with certain deficiency states and patients with known defects in lipid absorption so frequently exhibit low tocopherol levels, that it might be profitable to determine the effect of tocopherol on patients exhibiting these low values in an effort to uncover possible symptoms or metabolic derangements due to tocopherol deficiency. If tocopherol deficiency occurs in the human, it would be reasonable to expect it in such patients. On the other hand, until some positive effect of tocopherol is demonstrated in these patients, they cannot be classified as deficient in tocopherol on the basis of blood level alone.

It is realized that the present study is but a preliminary survey of the variations of tocopherol levels in health and disease. It seems to us, nevertheless, to point to the necessity for further investigations of the significance of hypertocopherolemia and its relationship to hypercholesterolemia and

other evidences of deranged lipid metabolism in human disease. In addition, the significance of hypotocopherolemia and possible metabolic evidences of tocopherol lack in patients who are malnourished due to dietary restriction, or to difficulties of gastrointestinal absorption, must be explored.

Summary

The concentration of total tocopherols in the plasma has been measured in healthy subjects and patients with medical diseases. The observations permit the following generalizations:

(1) Lower-than-usual values are commonly encountered in those diseases characterized by impaired absorption of fat—sprue, biliary obstruction, idiopathic steatorrhea, and fibrocystic disease of the pancreas.

(2) Unusually high values are associated with metabolic disturbances characterized by hypercholesterolemia. Such values have been observed in diabetes with nephrosclerosis, in carcinoma of the pancreas, carotenemia, in coronary occlusion, *etc.*

(3) Moderately high values are frequently noted in hypertensive cardiovascular disease.

(4) Plasma tocopherol levels rise during pregnancy, paralleling the observed increases in serum carotenes.

(5) A group of unsupplemented patients with coronary disease had levels similar to a control group of non-cardiacs.

(6) No regular variation of plasma tocopherol level could be correlated with a given phase of the menstrual cycle.

(7) In patients there was observed a slight positive correlation between tocopherol level and age. This specific effect of age cannot be separated, at present, from an influence of an increased frequency of diseases associated with hypercholesterolemia in the older patients.

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Discussion of the Paper

DOCTOR P. GYÖRGY (*School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania*): The absolute amount of tocopherol in the body fluids, including plasma, may not be proportional with its biological

activity. From the studies of our laboratory presented in this monograph it became apparent that the biological activity of tocopherol, for instance its protective effect on hemolysis, depends on the colloidal composition of the tocopherol-containing experimental fluid. Tenfold concentration of tocopherol in serum has been found inactive in comparison to water-dispersible tocopherol in saline solution.

Apparently, there must exist protein-tocopherol compounds, and perhaps tocopherol-fat (cholesterol) mixtures, the latter, perhaps, in analogy to carotene-fat mixtures (Josephs).

Total tocopherol values may be just as inconclusive as total calcium in the serum. Methods should be found which will help to assess the biologically active portion of total tocopherol.

OBSERVATIONS ON A BIOLOGICALLY ACTIVE VITAMIN E DERIVATIVE PRESENT IN HOG GASTRIC MUCIN AND IN HOG STOMACH LINING. THE BIOLOGIC ACTIVITY OF DL, ALPHA-TOCOPHERYLHYDROQUINONE*

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AND W. E. BARTELS

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Introduction

Early in the course of our investigations on the utilization of vitamin E in patients with progressive muscular dystrophy, we noted that alpha-tocopherol or its esters, administered either orally or intra-muscularly, were without effect on the chemical abnormalities and the clinical symptoms.¹ This is in striking contrast to the effect in animals with muscular dystrophy produced by deprivation of vitamin E. However, when alpha-tocopherol was ingested by a normal subject, and the contents of a gastric expression, performed one-half hour later, were administered to patients, decrease in creatinuria sometimes occurred.² This suggested the need either of some gastric substance in the utilization of alpha-tocopherol or of some necessary alteration in the vitamin E molecule occurring in the gastro-intestinal tract, although the possibility of similar reactions occurring at sites elsewhere in the body could not be disregarded.

These observations on donor-feeding experiments suggested further that, in patients with progressive muscular dystrophy, there is a defect in the utilization of vitamin E.³ That this defect is not merely one of absorption of the vitamin from the gastro-intestinal tract is indicated by the finding⁴ that the amounts of tocopherol in the blood of such patients are not unusual.

Many aspects of gastro-intestinal function were investigated, and preparations derived from organs concerned with these functions were studied for their possible effect on the utilization of tocopherol.

Our attention was directed particularly to gastric mucin, since certain carbohydrates, namely, d-galactose, d-mannose, and l-fucose contained in the polysaccharide portion of mucin, were observed to influence the phenomenon we were investigating. Thus, while neither tocopherol nor any of these sugars alone had any effect on the creatinuria of patients with progressive muscular dystrophy, the simultaneous administration of alpha-tocopherol and any of these sugars decreased the creatine output in suitable patients.⁵

Observations with gastric mucin were carried out on patients maintained on a constant creatine-free diet. One patient with progressive muscular dystrophy of the type of Landouzy-Dejerine received the identical diet every day for a period of over 3 years. The response of this patient to various test substances was considerably greater than was that of other patients. The administration of 12 gm. fresh commercial hog gastric mucin together with 500 mg. alpha-tocopherol daily for 3 days significantly lowered the

* Aided by the Armand Fund for Research in Muscular Disease and by a grant from the Nutrition Foundation, Inc.

creatinuria for periods of from 11 to 16 days. This effect at first was thought to be related to the carbohydrates contained in the hog gastric mucin.⁶ However, determination of the group-specific substances in the gastric mucin of patients, by methods based on technics used for blood grouping, disclosed no differences between the mucin of a large series of patients and that of normal subjects. Moreover, samples of commercial hog mucin, which, when fresh, showed high biologic activity, were without effect on creatinuria after the mucin had been kept at room temperature in the laboratory during the warm summer months. This observation suggested the experiment in which an active sample of mucin was heated under conditions that would have no effect on any of the tocopherols, and certainly not on the carbohydrates in mucin. Administration of the heated mucin together with alpha-tocopherol did not affect the creatine output of a patient in whom the unheated sample had a pronounced effect. The demonstration that fresh gastric mucin without the tocopherol supplement decreased the creatinuria as much as did the same sample of mucin when given with tocopherol made it appear likely that the biologically active principle in the mucin is a Vitamin E substance, but one which, because of its lability, is not any of the tocopherols.*

Experimental

More direct evidence on this point was obtained when, by use of a modification of the method of Kaunitz and Beaver,⁷ mucin was extracted with acetone and the acetone-soluble fraction was subsequently extracted with hexane. The acetone-insoluble fraction of mucin was without effect on the creatinuria of a patient, whereas the acetone-hexane soluble fraction promptly lowered the creatine output (FIGURE 1). This latter fraction

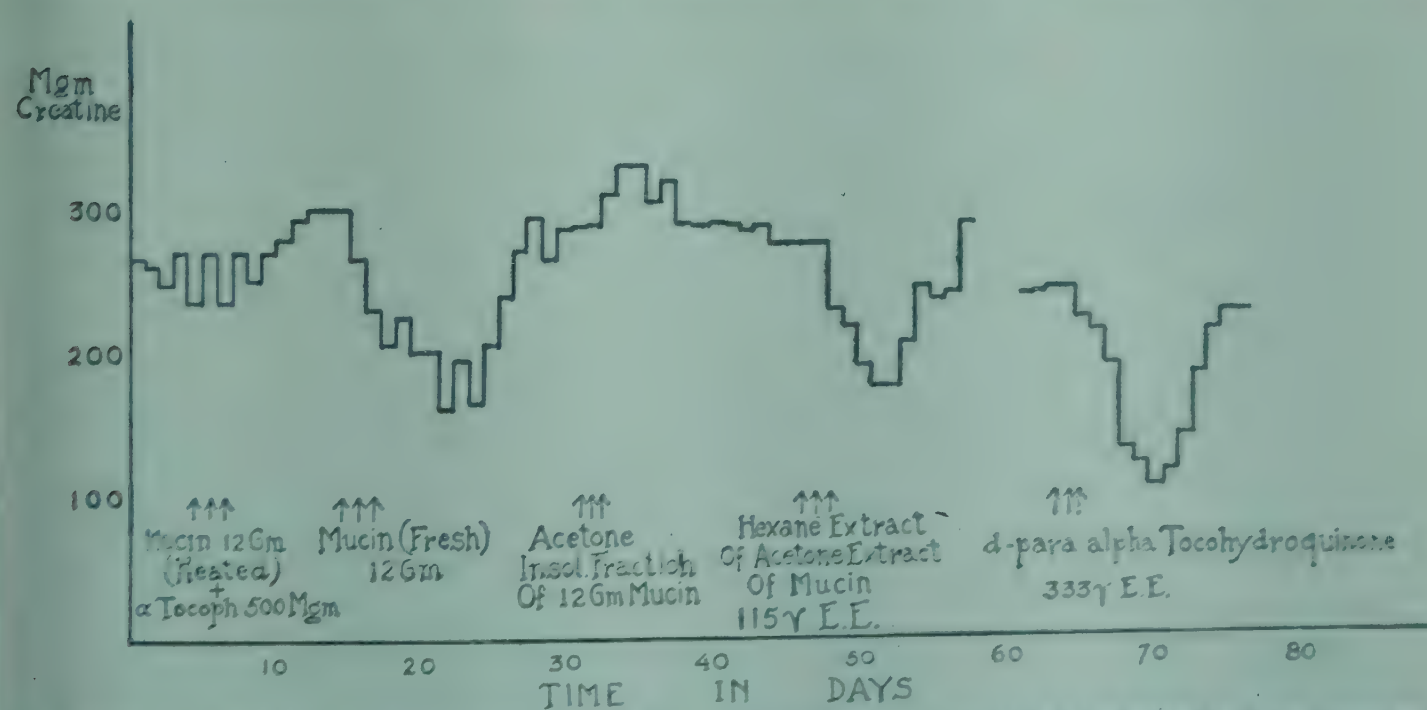


FIGURE 1. The effect on creatinuria of fresh hog gastric mucin (or treated as indicated), of an extract of the mucin, and of an alpha-tocopherylhdroquinone. The patient had progressive muscular dystrophy of the Landouzy-Dejerine type and was maintained on a constant diet low in creatine.

contained a substance that gave an Emmerie-Engel reaction. The residue of the acetone-hexane extraction administered daily for 3 days contained

* Acknowledgment is made to Winthrop Stearns, Inc. for supplies of hog gastric mucin, to the Armour Laboratories for preparing acetone extracts of hog stomach linings, and to Distillation Products, Inc., Merck & Co. Inc., and Hoffmann La Roche Inc. for gifts of tocopherols and their derivatives.

115 γ of Emmerie-Engel reacting substance, calculated as alpha-tocopherol—an amount equivalent to that estimated to be in the amount of mucin (12 gm.) given daily in the previous experiments.

The first indication of the chemical nature of the biologically active substance found in normal human gastric expressions and in hog gastric mucin came from an experiment in which we succeeded in regenerating the activity of hexane extracts of acetone extracts of gastric mucin which had lost their activity after storage in the laboratory over the summer months.

We noted that these inactive extracts had a reducing action equivalent to 0.1 mg. alpha-tocopherol per 100 grams gastric mucin, as measured by the Emmerie-Engel reaction, and that they could be treated with stannous chloride in the presence of concentrated mineral acid, according to the procedure of Tischler and Wendler,⁸ to produce an increased and stable reducing action equivalent chemically to 1.4 mg. alpha-tocopherol per 100 grams of gastric mucin. This suggested a tocopherylquinone whose formation was related to the loss of activity of the gastric mucin. This tocopherylquinone may have arisen from a tocopherol or some other tocopherylquinone precursor. The cyclized extracts were inactive. Activity was regenerated, however, when another aliquot of the hexane extract was treated with stannous chloride in the presence of dilute mineral acid. The reducing action was increased to that of only 0.94 mg. of alpha-tocopherol per 100 grams of mucin. The reducing action did not remain at this value but fell to a value equivalent to 0.40 mg. of alpha-tocopherol on the second day, to 0.35 mg. on the third day, and reached a value equivalent to 0.27 mg. by the end of a week. This lack of stability resembled that of the active substance in fresh gastric mucin. A study of the same reactive conditions applied to synthetic alpha-tocopherylquinone showed that the first set of conditions resulted in reduction plus ringclosure, whereas in the second experiment only reduction to the tocopherylhydroquinone occurred, which, on standing, reverted to the original tocopherylquinone. That the regenerated activity in gastric mucin could be duplicated by a tocopherylquinone was confirmed by the assay of synthetic alpha-tocopherylhydroquinone when administered to the patient in amounts of 0.33 mg. per day for 3 days. All of the 4 tocopherols, when given at these levels, had been found to be without effect in this patient.

It is interesting to recall that, in 1945, Dr. J. Baxter of Distillation Products, Inc. made analyses on biologically active samples prepared by us from alpha-tocopherol by two different methods.² He found the samples to have an absorption maximum at 265 μ and suggested that the active substance might be an oxidation product of alpha-tocopherol. Dr. Baxter very kindly prepared both alpha-tocopherylquinone and a mixture of alpha- and gamma-tocopherylhydroquinone, which we tested and found to be biologically inactive in patients. Unfortunately, the tocopherylhydroquinone was administered as the triacetate, and we since have found the triacetate, diacetate, and diphosphate to be without activity in the patient and in animals, although the free alpha-tocopherylhydroquinone is active. Possibly, these esters are oxidized during the process of hydrolysis in the body.

The biological activity of dl, alpha-tocopherylhydroquinone was tested in animals according to the procedure employed by Mackenzie and McCollum⁹ in their discovery of the antidystrophic action of alpha-tocopherol in the rabbit. Young rabbits weighing from 500 to 700 gm. were allowed to develop a stage II-III dystrophy and a creatinuria of approximately 100 mg. per day. The dl, alpha-tocopherylhydroquinone dissolved in a 10 per cent alcohol-90 per cent propylene glycol mixture was then administered intravenously and the effect on growth, food consumption, physical symptoms, and creatine excretion was observed.

The response of the dystrophic rabbit, as measured by these criteria, to single intravenous doses of from 5 to 50 mg. of dl, alpha-tocopherylhydroquinone was prompt and dramatic. These doses produced an 80 per cent drop in creatine excretion, an increase in food consumption, a gain in weight, and an improvement in or disappearance of physical symptoms. The creatine excretion was depressed for approximately 6 days irrespective of the amount of tocopherylhydroquinone injected. This was in sharp contrast to the graded response reported by Mackenzie and McCollum¹⁰ following the oral administration of dl, alpha-tocopherol and suggested that the dl, alpha-tocopherylhydroquinone was being destroyed (or excreted) at a rather rapid rate.

Accordingly, rabbits with acute dystrophy were injected intravenously with from 7 to 14 mg. of dl, alpha-tocopherylhydroquinone daily. The creatine excretion fell to a normal level, growth increased rapidly, and all physical signs of dystrophy disappeared completely. Microscopic lesions were absent from an animal sacrificed one week after the beginning of therapy. It may be concluded, from these results, that dl, alpha-tocopherylhydroquinone is effective in curing all of the symptoms of experimental muscular dystrophy.

A reasonable formulation is that tocopherylhydroquinone cannot be stored by the body, whereas alpha-tocopherol can be stored as such and is converted as needed to a biologically active form, probably the tocopherylhydroquinone.* Patients with progressive muscular dystrophy appear to have a defect in this mechanism of conversion. The conversion of alpha-, beta-, gamma-, and delta-tocopherol to their hydroquinones is not equally deficient in progressive muscular dystrophy, since both beta- and delta-tocopherol can reduce creatinuria when given at high dosage levels (*e.g.* 200 mg. daily), whereas alpha- and gamma-tocopherol were found to be inactive.

Preliminary observations suggest that, when given in amounts in which the tocopherol itself is inactive, the hydroquinone of delta-tocopherol can reduce the creatinuria of both patients and rabbits with muscular dystrophy. More experiments are required and now are being carried out, but the observations, while not yet definitive, suggest that the relatively low potency of delta-tocopherol in normal animals may be related, at least in part, to greater difficulty encountered in the conversion of the tocopherol to its hydroquinone. In a patient in whom either 9 mg. of δ -tocopherol or 330 mg. of γ -tocopherol per day for 3 days was without effect, a daily dose

* More recently, alpha-tocopheramine hydrochloride has been tested in both animals and a patient with muscular dystrophy. The potency in rabbits with muscular dystrophy is about equal to that of alpha-tocopherol and, in the patient, 9.6 mg. administered daily for 3 days lowered the creatine output.

either of 2 mg. δ -tocopherylhydroquinone or of 11 mg. γ -tocopherylhydroquinone for a similar period significantly lowered the creatinuria.

Finally, a biologically active substance, similar to that found in hog gastric mucin has been demonstrated in hog stomach linings.

Addendum

S. ULICK: The question of the purity of the alpha-tocopherylhydroquinone used in these studies should be considered, since it is a readily oxidized substance prepared from alpha-tocopherylquinone, preparations of which may retain vitamin E activity unless carefully purified.

Destruction by atmospheric oxidation was minimized by using freshly hydrogenated solutions of alpha-tocopherylquinone in absolute ethanol for patients and in propylene glycol for animals. At the time of administration, each preparation was assayed for tocopherylhydroquinone content by the Emmerie and Engel method, and for tocopherylquinone content by measurement of the absorption at $270\text{ m}\mu$. The tocopherylquinone used contained less than 0.25 per cent alpha-tocopherol and had an $E^{1\%}$, ($270\text{ m}\mu$) of 440 ± 10 (FIGURE 2). The preparations were free from 3 other possible impurities which may be formed in the preparation of alpha-tocopheryl tocopherylquinone by the oxidation of alpha-tocopherol, the ortho-quinone derived from alpha-tocopherol and materials designated compounds 1 and 2. The ultra-violet absorption spectra are shown in FIGURE 2. Compound 1 is formed with ferric chloride as the oxidizing agent and compound 2 with gold chloride. Both compounds 1 and 2 can be further oxidized to tocopherylquinone by further treatment with gold chloride and both are best formed in the two phase system, iso-octane-absolute methanol, which helps to remove the product from the oxidizing agent as it is formed. The spectra shown in FIGURE 2 were determined after the materials had been purified by counter-current distribution. Compound 1, with a single maximum absorption at $238\text{ m}\mu$, is apparently the same as the substance discussed by Dr. Boyer previously in this monograph and which he characterized more completely as an epoxychroman. Compound 2 has not been described previously. It differs from compound 1 in that it retains the tocopherol type of spectrum with a shift to the longer wavelengths and in that it gives the Emmerie-Engel reaction, although at a rate approaching that of delta-tocopherol. Compound 2 did not reduce the creatinuria of the patient. A comparison of the partition coefficients, in the iso-octane-methanol two phase system, of the substances formed in the oxidation of alpha-tocopherol is shown in TABLE 1.

Addendum

H. ROSENKRANTZ: Infra-red absorption spectra were obtained for differentiation of the tocopheramine, the tocopheryl quinones and the tocopheryl hydroquinones. Absorption bands between 8 and $12\text{ m}\mu$ distinguish not only the tocopherols, but also tocopheramine. In addition to the absorption in this region, absorption bands around $6\text{ m}\mu$ easily differentiate the tocopherylquinones from the tocopherols. Characteristic

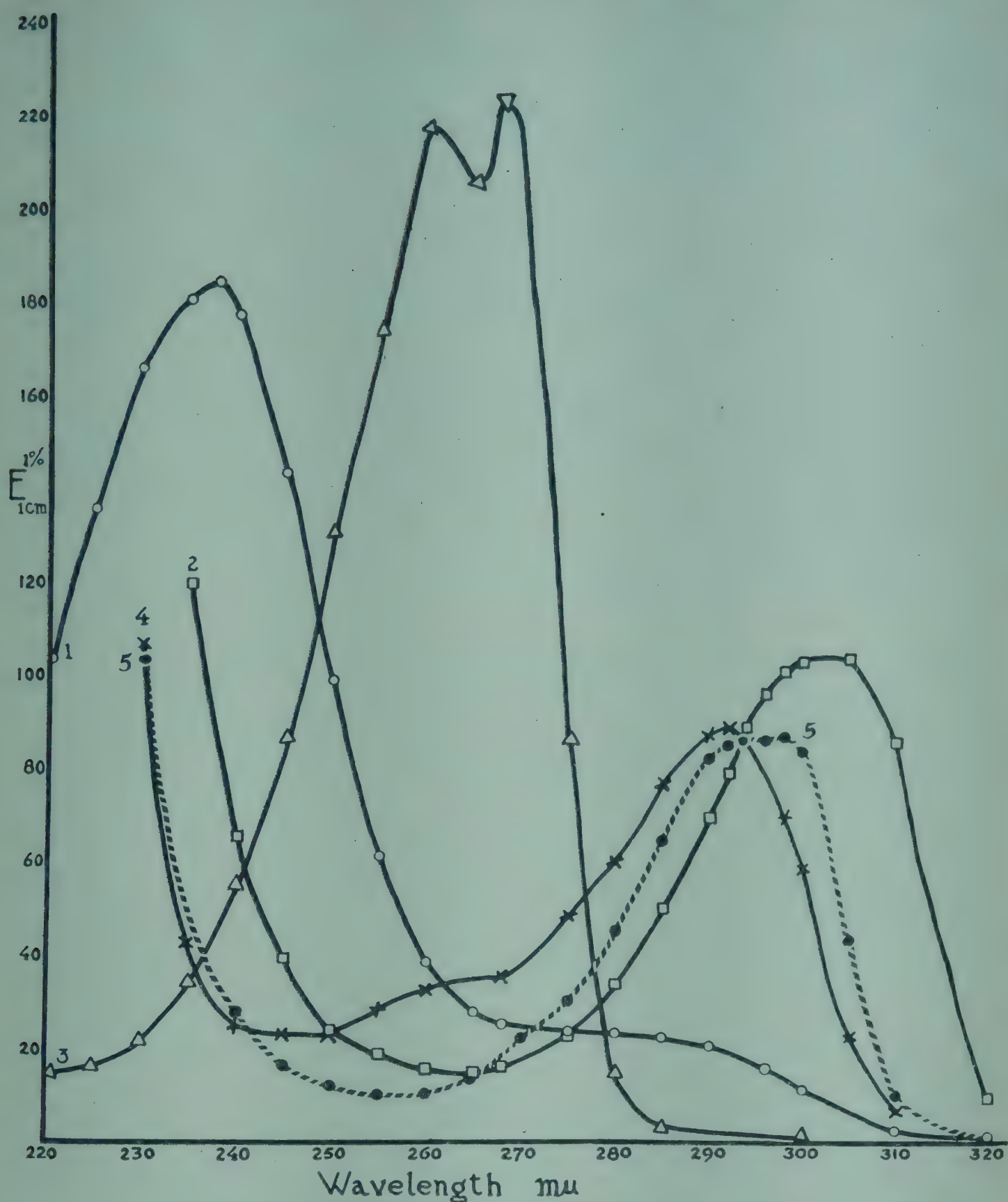


FIGURE 2. Ultra-violet absorption spectra of oxidation products of alpha-tocopherol: 1. Compound 1 ($\times \frac{1}{2}$); 2. Compound 2; 3. dl, alpha-tocopherylquinone ($\times \frac{1}{2}$); 4. dl, alpha-tocopherylhydroquinone; 5. dl, alpha-tocopherol.

TABLE 1

PARTITION COEFFICIENTS OF OXIDATION PRODUCTS OF ALPHA-TOCOPHEROL IN AN ISO-OCTANE-METHANOL TWO-PHASE SYSTEM

Substance	K
alpha-tocopherol	1.4
alpha-tocopherylquinone	0.8
alpha-tocopherylhydroquinone	0.15
Compound 1	4.3
Compound 2	2.4

bands in both these regions identify also the tocopherylhydroquinones. The purity of the alpha-tocopherylquinone used in these investigations was ascertained also through infra-red studies.

A sample of alpha-tocopherylhydroquinone has been crystallized from ethanol at room temperature.

Summary

(1) An acetone-soluble, hexane-soluble fraction of gastric mucin decreased creatinuria when administered orally to a patient with progressive muscular dystrophy.

(2) Activity of this fraction was easily destroyed by oxidation and alpha-tocopherylquinone was identified among the oxidation products.

(3) d,l alpha-tocopherylhydroquinone reduced creatinuria in patients with muscular dystrophy and also cured muscular dystrophy in standardized, vitamin E-deficient rabbits.

(4) d,l alpha-tocopherylhydroquinone was prepared in crystalline form from synthetic alpha-tocopherol.

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V

VITAMIN E IN CLINICAL MEDICINE: INTRODUCTORY REMARKS

By ROBERT S. GOODHART

I am grateful for the privilege accorded me of presiding at today's session of this International Conference on Vitamin E.

During the preceding sessions we have been treated to excellent critical expositions of the chemistry and the biology of the tocopherols—discussions that involved analyses of both past and current investigative work.

It became apparent as the Conference progressed that there are serious deficiencies in our knowledge of the biology of the tocopherols. These deficiencies are of a nature so as to require that present research on the clinical applications of vitamin E be largely empirical. Certainly, a number of leads, such as the inhibitory effects of vitamin E upon hyaluronidase activity, have been developed by the biologists and the biochemists to guide the clinician, but the definition of the place and value of vitamin E in clinical medicine remains to be determined by the process of trial and error in a variety of clinical conditions. This being the case we may expect our discussions today, on the clinical applications of vitamin E, to involve an apparent miscellany of diseases and affections of man.

The organizers of the Conference have done a splendid job in providing us with speakers who represent the most active and outstanding workers in vitamin E research. As today's discussion progresses you will find conflicting viewpoints expressed, an indication of the complexity of clinical research and of our inadequate knowledge of the biology of vitamin E. I know that our speakers hope and anticipate that there will be full and uninhibited discussion from the floor. That is the purpose of the Conference, to promote discussion, clarify issues and, most of all, to stimulate much needed clinical research work.

RELATIVE BIOLOGICAL POTENCY OF VARIOUS TOCOPHEROLS USED IN THERAPEUTIC PREPARATIONS OF VITAMIN E

By PHILIP L. HARRIS

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All of the vitamin E preparations which are available in the United States for the physician to prescribe or for the individual consumer to purchase are made from the source materials shown in TABLE 1. These materials are processed into convenient forms for use—capsules, tablets, ampoules, *etc.*—by numerous pharmaceutical companies. The finished products are frequently labeled with trade names which furnish little or no information concerning the type of vitamin E used. Consequently, the fine print on the label should be read by the prescriber or consumer.

Synthetic preparations of vitamin E are of two kinds, dl, α -tocopherol and dl, α -tocopheryl acetate, and have been available for many years.

Concentrates of mixed tocopherols, from natural sources, prepared by molecular distillation have been available for about nine years. Recently, concentrates of d, α -tocopheryl acetate, also from natural sources, have become widely used. Wheat-germ oil, a source of natural vitamin E of very low concentration, is still marketed, although it is primarily of historic interest as the vegetable oil richest in vitamin E content.

Use

Preparations made from mixed tocopherol concentrates are available for those who are interested in using all of the natural tocopherols. In these, half of the tocopherol is d, α -tocopherol and the other half is a mixture of the other three natural tocopherols, β -, γ -, and δ -tocopherols. Vitamin E occurs throughout nature as mixtures of the free tocopherols. It is in the free form that tocopherols exert a sparing effect or antioxidant activity both *in vitro* and *in vivo*.

Preparations prepared from synthetic dl, α -tocopherols and from d, α -tocopheryl acetate concentrates are available for those who consider α -tocopherol to be the effective form of vitamin E in nutrition and in therapy. Although acetylated tocopherols have no antioxidant activity and apparently do not occur in nature, α -tocopheryl acetate is used therapeutically because of its superior biopotency and stability relative to free α -tocopherol.

Potency

The various α -tocopherol preparations differ in their biological potency as determined by the standard rat-bioassay technique.¹ The relative physiological activities of the various tocopherols are shown in TABLE 2.² These data show that d, α -tocopherol is more potent than dl, α -tocopherol and that the esters are more active than the free tocopherols. For easy comparison of the vitamin E potency of pharmaceutical preparations containing different types of tocopherols, International Units may be used as a common denominator of biological activity. Thus, if a capsule contained 100 mg.

TABLE 1
SOURCE MATERIALS FOR THERAPEUTIC PREPARATIONS OF VITAMIN E

Label Description		Vitamin Content
Synthetic	“α-tocopherol”	100% dl, α-tocopherol
	“α-tocopherol acetate”	100% dl, α-tocopheryl acetate
From Natural Sources	Concentrate of mixed tocopherols:	34% tocopherols— 17% d, α-tocopherol 17% other tocopherols
	Concentrate of d, α-tocopheryl acetate:	25% d, α-tocopheryl acetate A few % of other tocopheryl acetates
	Wheat germ oil:	0.20% tocopherols—approx. 0.12% d, α-tocopherol 0.08% d, β-tocopherol

TABLE 2

Form of vitamin E	Equivalency (Int. Units/mg.)	Calculation
dl, α-Tocopheryl Acetate	1.00	by definition of I.U.
dl, α-Tocopherol	0.68	$1.00 \div 0.91 \text{ (a)} \div 1.62 \text{ (b)} = 0.68$
d, α-Tocopherol Acetate	1.36	(c)
d, α-Tocopherol	0.92	$1.36 \div 0.91 \div 1.62 = 0.92$

(a) 0.91 = ratio of molecular weight of α-tocopherol to that of α-tocopheryl acetate.
(b) 1.62 = ratio of activity of α-tocopherol, as an ester, to that of α-tocopherol as determined by bioassay.
(c) 1.36 = ratio of activity of natural α-tocopherol to that of synthetic α-tocopherol as determined by bioassay.

of synthetic dl, α-tocopheryl acetate, it would supply 100 International Units, since, by definition, one I. U. is equal to the potency of one mg. of dl, α-tocopheryl acetate. Also, a capsule containing 100 mg. of synthetic dl, α-tocopherol would furnish only 68 I. U. of vitamin E. A capsule containing 100 mg. of natural d, α-tocopheryl acetate supplies 136 I. U. and one containing 100 mg. of natural d, α-tocopherol furnishes 92 I. U. of vitamin E. Using these potency relationships, physicians can readily compare or set dosage levels of vitamin E in their clinical practice or research, even though different therapeutic preparations are administered.

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Discussion of the Paper

MR. SERECK H. FOX: (Gelatin Products Div., R. P. Scherer Corp., Detroit, Mich.) I would suggest, in view of the rather confusing situation which was disclosed by TABLE 2 of Dr. Harris's paper, confusing at least to the physician and to him to whose lot it falls to write label copy, that it behooves

all of us in the pharmaceutical industry to produce label copy which in no way misrepresents the subject. Further, the biological potency of the preparation should be described in terms of a single standard, preferably the International Standard, in order that all such products shall be on the same potency basis, and so that clinicians may be well informed for the purpose of planning dosage.

Dr. Harris's disclosure, that the esterified forms of tocopherols have a greater biologic effect than do the free tocopherols, is an interesting point. Recently it has been found, in certain work with vitamin A, that, depending on the fat content of the rat diet, the relative superiority of the free forms of vitamin A *versus* esterified forms is reversible. In the high fat diet, it would seem that vitamin A alcohol produces a greater growth response. In a low fat diet a vitamin A ester will produce a greater response than will vitamin A alcohol.

The possible reversibility of the apparent biologic potencies of free tocopherols and tocopheryl acetates, depending on the fat content of the rat diet, should be investigated.

COMPARISON OF EFFECTS OF ALPHA-TOCOPHEROL AND A MATCHING PLACEBO ON CHEST PAIN IN PATIENTS WITH HEART DISEASE*

By JANET TRAVELL, SEYMOUR H. RINZLER, HYMAN BAKST, ZACHARY H. BENJAMIN, AND AUDRIE L. BOBB

The Cardiovascular Research Unit of Beth Israel Hospital, and the Department of Pharmacology, Cornell University Medical College, New York

We were led to study the effects of vitamin E on chest pain in patients with heart disease because of our interest in what we have called the "somatic component" of cardiac pain.^{1, 2, 3} In extension of the work of Weiss and Davis,⁴ we had shown that spasm of the chest muscles results from impaired coronary flow and may cause persistent pain which responds to local treatment of the voluntary muscles themselves.^{1, 2, 3}

During the course of these studies, it occurred to us that the reported relief^{5, 6, 7, 8, 9} of effort angina by alpha-tocopherol might be due not solely to an action of the vitamin on the heart, but partly to an action on the skeletal muscles of the chest, which we know may participate in the anginal syndrome. This hypothesis was supported by the fact that animals on E-deficient diets may show reversible changes in both cardiac and skeletal musculature,^{10, 11, 12} and by reports on the clinical relief of pain by vitamin E in such disorders as fibromyositis.^{13, 14}

We undertook, therefore, to assess by the blind-test method the effect of alpha-tocopherol on chest pain in cardiac patients by means of parallel series of vitamin-treated and placebo-control cases. At that time, no negative reports had been published, and we were hopeful of elucidating the mechanism of action of vitamin E on the somatic and visceral components of chest pain.

Methods

During a preliminary period, 41 patients with long-standing chest pain and with either arteriosclerotic or hypertensive heart disease, or both, were selected from the case-load of the cardiac clinic. About 75 per cent of these patients had effort angina. The remainder had intermittent pain unlike effort angina (not induced by walking and not relieved promptly by rest or nitrites) and constant chest pain. We regard the first type as primarily cardiac and the last two as having a large somatic component.

After the necessary base-line observations had been made, the patients were paired to match as closely as possible with respect to pertinent factors. In order to "randomize" the parallel series, one of each pair was allotted by chance to the vitamin-treated, and one to the placebo-control group. TABLE 1 shows that the matching of patients was satisfactory and that the two groups were indeed closely comparable with respect to their initial status.

Blind-test methods were then applied both in the collection and interpre-

* "Ephynal Acetate" supplied by Hoffmann-La Roche, Inc.

TABLE 1
COMPARISON OF TREATED AND CONTROL GROUPS PRIOR TO MEDICATION

	<i>Alpha-tocopherol group</i>	<i>Placebo group</i>
	<i>no. cases</i>	<i>no. cases</i>
Total Cases	19	19
Sex: Males	9	13
Females	10	6
Age: Average	61 yrs.	59 yrs.
Range	(49-72)	(47-77)
Duration of chest pain: Average	6.1 yrs.	7.4 yrs.
Range	(1/5-15)	(1/6-15)
Effort angina	15	14
Somatic chest pain	4	5
Hypertension	6	8
systolic over 200 mm.	2	2
Previous myocardial infarction	9	7
Abnormal electrocardiogram	8	11
Congestive heart failure	0	0
Diabetes mellitus	1	2
Somatic pain syndromes (exclusive of chest)	12	13
Osteoarthritis of spine	19	19

tation of data. One person (S. H. R.) issued the medication and never examined the patients. Thus, none of the examiners knew which patients were receiving the vitamin and which ones, the placebo. Even judgments regarding the final result were made in each case without knowledge of the nature of the material administered.

Thirty-eight patients completed the course of medication. Of these, 19 received alpha-tocopherol and 19, the placebo. Of the original 41 patients, 3 in the vitamin-treated group refused to continue the medication on account of increased chest pain and had to be dropped from the series.

Synthetic alpha-tocopherol acetate ("Ephynal Acetate") was used. The dosage was 200 mg. daily for two weeks and then 300 mg. daily, given by mouth in divided doses of 100 mg. each. The same number of matching placebo tablets was prescribed for the control group.

The vitamin was administered for an average of 16 weeks (10 to 20 weeks), and the placebo for an average of 16.6 weeks (10 to 20 weeks). Since Vogelsang, Shute, and Shute¹⁵ report that the optimal dose of alpha-tocopherol is about 200 mg. daily and that this will usually relieve anginal pain in 5 to 10 days, the amounts of the vitamin which our patients received over

periods of 2.5 to 5 months may be regarded as representing an adequate clinical trial.

During the investigation, the use of iron preparations and liquid petrolatum was prohibited. Nitroglycerine, maintenance doses of digitalis, and other medications were continued as before. None of the patients were receiving thyroid.

A total of 365 visits to the clinic was made by the 41 patients, exclusive of those for laboratory tests. At each visit, usually at intervals of 2 to 3 weeks, the status of pain was evaluated, a routine cardiovascular examination was done, and, in addition, each patient was examined for tender spots, or "trigger areas,"^{2, 16} in the chest muscles.

Exercise tolerance tests by the Master two-step technic¹⁷ and standard lead electrocardiograms were repeated at intervals during the investigation. Satisfactory exercise tolerance tests were obtained in 16 patients. In the remainder, either the end-point was indecisive or the onset of pain in the legs terminated the test before chest pain appeared.

Two measurements of skeletal muscle function were made at each visit: (1) muscle strength and (2) muscular endurance during ischemia. As an index of muscle strength, the grip of each hand was measured by means of a spring dynamometer. Muscular endurance during ischemia¹⁸ was measured by the number of times the subject could open and close the fingers at a rate of 30 isotonic contractions per minute, while the circulation was occluded by a blood pressure cuff. The technic was modified slightly from that used by Lewis¹⁹ in studies on ischemic pain.

Results

Chest Pain. The response to medication was essentially the same for vitamin-treated and placebo-control groups. No improvement was noted in 12 treated subjects (63 per cent) and 14 controls (73 per cent). Partial relief of pain was reported by 7 treated subjects and 5 controls. Thus, subjective improvement occurred in 37 per cent of those who received the vitamin and in 27 per cent of those who received the placebo. These figures agree closely with those of Evans and Hoyle,²⁰ who reported that the administration of a placebo to patients with angina pectoris was attended by a diminution of pain in about 40 per cent of the cases.

Two of the patients who received the placebo developed intercurrent clinical complications. In one, congestive heart failure occurred after 4 weeks. The other sustained an acute myocardial infarction after 16 weeks on the medication. Since these complications occur spontaneously in the course of arteriosclerotic heart disease, it cannot be considered statistically significant in so small a series that both these cases happened to fall in the control group.

When we analyzed the data for different types of chest pain, the effect of medication with respect to cardiac and somatic components was similar for vitamin and placebo groups. Furthermore, significant changes in the trigger areas in the chest muscles during medication occurred in only 2 patients. These two became free of muscle tenderness by the end of the study; one received alpha-tocopherol and one, the placebo.

The statements of the 7 patients who considered their chest pain improved by alpha-tocopherol are presented in TABLE 2. It is evident that the

TABLE 2
ANALYSIS OF DATA IN PATIENTS WHO CONSIDERED THEIR CHEST PAIN
IMPROVED BY MEDICATION

<i>Type of chest pain</i>	<i>Patient's statement: basis of improvement</i>	<i>Change in exercise tolerance after medication</i>
<i>Alpha-Tocopherol</i>		
Effort angina	Can now walk 6 or 8 blocks without pain, formerly 2 blocks.	-70
Effort angina	Now walks same distance as before medication, but pain is less severe.	-12
Effort angina	Can walk much further without pain, now 46 blocks, formerly 2½ blocks.	+6
Effort angina	Attacks occur less often now.	-5
Effort angina	Can continue walking a little distance after onset of pain, whereas before he had to stop at once.	+20
Intermittent	Attacks now occur at longer intervals; pain just as severe during attacks.	Not done (leg pain)
Intermittent	Attacks occur less often and are less severe.	-17
<i>Placebo</i>		
Effort angina	Can now walk 12 blocks without pain, formerly 2 blocks.	+633
Effort angina	Attacks of pain are much less severe.	0
Effort angina	Can now walk 2 blocks without pain, formerly 1 block.	Test refused
Intermittent	Chest pain is less severe.	Not done (leg pain)
Constant, Effort angina }	Constant chest pain is gone. Pain occurs on walking as before.	+200

improvement is a matter of degree and that in no instance was there total relief of pain. Furthermore, the exercise tolerance test did not support the patients' testimony that they were better following medication. None showed a corresponding increase in this objective measurement of the work capacity of cardiac muscle. It may be seen (TABLE 2) that exactly the same kind of statements were made by the 5 patients who considered their chest pain improved by the placebo.

Of all the patients in either group who reported improvement, only two showed a significant increase in exercise tolerance, namely, 200 and 633 per cent, respectively (TABLE 2). Both of these patients received the placebo. The one with the 200 per cent increase had severe, constant chest pain which disappeared spontaneously and left him with effort angina alone. The patient with the 633 per cent increase had sustained an acute myocardial infarction 6 months before the first control exercise tolerance test, and it is probable that the final test, 5 months later, reflects merely the spontaneous improvement in myocardial function which often occurs during recovery from this acute episode.

In this connection, it should be noted that no patients were included in this study who had had an acute myocardial infarct within 6 months of the control observations. In 4 patients, infarction had taken place in from 6 months to one year before the start of the investigation. Three of these were in the vitamin-treated group, and one was in the placebo-control group. Thus, if recovery from a fairly recent infarction is a factor in the relief of chest pain and in improved myocardial function, any possible weighting of our results is in favor of the alpha-tocopherol group.

Cardiovascular Status. Serial electrocardiograms and serial blood pressure readings, like the exercise tolerance tests, showed no significant changes in relation to the administration of alpha-tocopherol. In no instance did an abnormal electrocardiogram become normal or tend to return toward normal. Appreciable lowering of the blood pressure occurred in only one patient with hypertension, and that one received the placebo; the readings at consecutive visits before medication were 210/120, 220/120, and 215/120 and, during the course of medication, they were 150/80, 185/102, 190/110, and 164/94. In the vitamin-treated group, significant changes in the blood pressure level were not observed, except in relation to the complications of congestive heart failure and acute myocardial infarction in 2 patients, as already described.

Skeletal Muscle Function. Evidences of improvement in the capacity for work of skeletal muscle as the result of alpha-tocopherol administration were also lacking (TABLE 3). The strength of the grip following medication

TABLE 3
INFLUENCE OF MEDICATION ON FUNCTION OF SKELETAL MUSCLE

		<i>Alpha tocopherol group</i>	<i>Placebo group</i>
Strength of grip	<i>No. cases</i>	19	19
	No. units before medication }	38 (19-59)	47 (22-62)
	Change at end of medication }	-5% (-40% to +32%)	+2% (-42% to +32%)
Endurance during ischemia	<i>No. cases</i>	19	18
	No. contractions before medication }	44 (27-92)	44 (20-86)
	Change at end of medication }	+14% (-48% to +96%)	+25% (-36% to +100%)

showed an average change of -5 per cent for the vitamin-treated group and +25 per cent for the placebo-controls. Muscular endurance during ischemia showed no important differences for the two groups. The average change in endurance was +14 per cent after alpha-tocopherol administration and +25 per cent after the placebo. The increased performance during medication is probably attributable to training.

Toxic Effects. No toxic effects were ascribed to the doses of alpha-tocopherol employed. Non-specific complaints, such as drowsiness, nausea, constipation, palpitation, and weakness, were blamed on the medication with equal frequency for the vitamin and placebo groups. In every instance, these symptoms disappeared during continued administration of the tablets. As we have mentioned, 3 patients insisted on stopping alpha-tocopherol because of increased chest pain. It was felt that this exacerbation of pain could not be attributed to the vitamin, since these individuals had been subject to such spontaneous attacks of severe pain prior to the medication.

Although it has been stated that the administration of more than 150 mg. of alpha-tocopherol daily to patients with hypertension may further raise the blood pressure,¹⁵ we did not observe such an effect after doses of 200 and 300 mg. daily, even in the 2 patients with systolic blood pressures of 210 and 220 mm. Hg. respectively. The initial dosage of alpha-tocopherol in 2 patients with hypertension (170/80 and 180/95, respectively) was 300 mg. daily.

Discussion

Because of the large number of variables which cannot be regulated in the clinical evaluation of therapeutic materials, each patient, ideally, should serve as his own control through alternation of active agent and placebo. This method, however, may lead to false conclusions when there is a carry-over of effects. Since vitamin E, like other fat-soluble vitamins, is stored by the body for considerable periods, we chose to set up parallel series, or matching groups of patients, to control our results. The wisdom of this choice is supported by the study of Donegan, Messer, Orgain, and Ruffin,²¹ in which the control blood tocopherol levels, just prior to the periods of vitamin administration, show an upward trend (the average level three months after the last dose of alpha-tocopherol was about 40 per cent greater than the control level before the first course of administration of this vitamin).

In clinical studies of drug therapy, the placebo control can be made to serve two purposes.²² Ordinarily, it is employed only to keep the patient in the dark. This enables one to measure the effects of the subject's psychological attitudes toward the medication and the physician. The second purpose is to keep the examiner in the dark, by allowing the study to be conducted under strictly blind conditions. The use of a placebo in this way obviates any possible weighting of the results by unconscious bias on the part of the observer. Even in animal experimentation, whenever judgments are involved, the blind-test control should be applied.²³

The extent of the placebo action and the need for this type of control in human subjects are well illustrated by studies on the effects of analgesics on pain²⁴ and of drugs on seasickness.²⁵ Nevertheless, the necessity for the placebo control is not yet universally recognized, and the essential character of the blind-test in clinical investigation is probably even less appreciated.

The lack of controls in the initial favorable reports on the effects of vitamin E in cardiac pain has been pointed out in recent comment^{26, 27} and probably explains, to a large extent, the discrepancies which appeared in the

literature when later investigators failed to duplicate these findings. In this connection, the negative results of Levy and Boas,²⁸ Baer, Heine, and Gelfond,²⁹ Makinson, Olesky, and Stone,³⁰ Ball,³¹ Ravin and Katz,¹² and Donegan, Messer, Orgain, and Ruffin²¹ should be mentioned.

The attitude of Vogelsang, Shute, and Shute toward the need for controls in such investigations is expressed in one of their papers published about a year ago. They state:³² "It should be remembered both that the number of cases we have studied is small and that our series is uncontrolled. The small numbers are to be ascribed to the fact that the authors have no access to hospitals, wards or to any facilities other than their private practices and one of them is a surgeon, one an internist, and one an obstetrician. This is not their fault, therefore, merely their misfortune." They go on to say that "Their series is 'uncontrolled' for the same reason," and they point out "that 'ideas' and 'controls' often seem to be incompatible." They say, "Those who have many patients on whom they may run extensive parallel series too rarely put their opportunities to creative or original use. . . . If one must choose he might forego the controls. . . . And have we not had too many centuries of pitiful cardiovascular 'controls'?"

Another difficulty may be the fact that Vogelsang, Shute, and Shute included in their series, on which evaluation of vitamin E therapy was based, patients with chest pain due to an acute or fairly recent myocardial infarction.⁵ For example, to quote from their case reports: "Case VII. Mr. C.... was first seen on January 4th. . . . January 19th, he had a typical attack of coronary thrombosis with persistent mild retrosternal oppression. . . . He was given 300 mg. tocopherex per day after January 20th. He made good progress, losing his anginal pain in twenty-four hours." Surely, the term "anginal pain" should not be applied to the pain of acute coronary thrombosis. This usage can lead only to the confusion of two separate clinical entities, namely, effort angina and myocardial infarction. Without controls, such cases are not suitable for evaluation of drug therapy, because, as we have noted, dramatic improvement in cardiac function may occur spontaneously at varying intervals after infarction. An illustration of this is the patient in our placebo-control group who had a large increase in exercise tolerance in the second 6 months following an acute infarct.

Conclusion

Our data on the effects of alpha-tocopherol acetate and a matching placebo in parallel series of patients afford no basis for the use of vitamin E in cardiac pain.

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Discussion of the Paper

DRS. M. E. EISEN AND H. GROSS (*City Hospital, New York, N. Y.*):
We have treated 52 patients with various types of heart disease and/or

peripheral vascular disease for periods of over a year with 150 to 800 mg. of vitamin E daily. This therapy produced transitory clinical improvement in many patients when they were advised that they were receiving a new medicine. When placebos were substituted the patients still claimed to feel better for a short period of time. However, when vitamin E was continued, improvement in both subjective and objective symptoms disappeared. All patients soon complained of all of their original symptoms.

Electrocardiographic studies before and after exercise with and without vitamin E therapy showed no significant differences. In the combination of coronary and peripheral arteriosclerotic disease in the same individual, patients claimed improvement in the anginal syndrome up to a period of six weeks, but no response in leg pain.

In the cases of heart disease, peripheral vascular disease, or a combination of both, the electrocardiograms revealed either no change or further impairment while the patients were taking vitamin E over a period of one year.

DOCTOR A. VOGELSANG (*London, Ontario, Canada*): I do not understand why more of Dr. Travell's patients with cardiac pain did not respond to treatment with alpha-tocopherol. The dosage used was almost adequate and might have been continued for a longer period of time. Another possible explanation of the difference between the results obtained by Dr. Travell and those obtained by me might be in the nature of the vitamin E preparations employed. The one I used was designed to be released and absorbed in the intestinal tract rather than in the stomach. The error in the investigations of Makinson, Levy, and Boas and Baer, Heine, and Gelfond rests in the fact that none of these investigators realized that vitamin E is not a substitute for, but is a supplement to, conventional cardiac therapy. The dosage used by these investigators was also inadequate and, in some cases, the period of administration was too brief.

PRECAUTIONS IN THE USE OF ALPHA-TOCOPHEROL IN THE TREATMENT OF HYPERTENSIVE HEART DISEASE

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It is a characteristic of most forms of successful treatment that the agent used is (1) dangerous in excess, (2) impotent in less than threshold dosage, and (3) most successful when the dosage used is fitted to the individual case. This is true of the use of morphine for the alleviation of pain, the use of the sulfonamides to combat infections, and the use of insulin in the treatment of diabetes mellitus. Giving 50 units of regular insulin to every diabetic one treated would not be considered wise therapy today, and similarly, ever since our experience in the treatment of heart disease passed beyond its initial stages, we have recognized that the dosage of alphatocopherol varied with each type of heart disease and with each case within that type. As a result, our paper read before the Academy of Medicine at Kansas City in April, 1947, contained a definite schedule of dosage used by our group. Further experience has confirmed the accuracy of that schedule.

In the treatment of hypertensive heart disease, large doses of alphatocopherol (300 or more mg. daily) administered from the beginning of treatment often cause a rise—which may be very considerable—in the systolic and diastolic pressure. This elevation may be maintained as long as the large dose is continued. Less often, the blood pressure remains at its original level, and the large dose may or may not lead to a disappearance of all the symptoms of cardiac involvement in the patient, whether they include angina pectoris, dyspnea on exertion, or other evidences of cardiac failure. Much less frequently, the response to large dosage is a fall in blood pressure, with or without disappearance of symptoms.

Small initial dosages of 75 to 100 mg., on the other hand, may lead to a lowering of blood pressure. As this lowering of both systolic and diastolic pressure develops, the dosage of alphatocopherol usually may be increased slowly until full dosage with remission of symptoms and signs occurs. The speed with which the dose may be increased safely varies widely in different, although clinically similar, cases. There are patients where a high dosage is never tolerated, as the blood pressure begins to rise again after a certain intake is reached. As suggested below, these people may have to compromise on dosage.

It is worthy of mention that where angina or dyspnea on exertion is the chief symptom, it may disappear soon after the pressure has begun to fall, even on relatively small dosage. The aim of treatment in hypertensive heart disease is generally a daily intake of at least 300 mg. of alphatocopherol. We do not like to see the patient stop short of that amount for maximum relief and the best prognosis. The best compromise is probably a reduced pressure plus a loss of symptoms at a lower dosage level, adjusted to both blood pressure and cardiac relief.

We have used many vasodilators such as nitrite compounds and the xanthine derivatives, pancreatic extracts such as Depropanex, and sedation

as adjuncts to the tocopherol treatment of hypertensive heart disease, but without any apparent success. Eventually, a large number of cases have had to be considered failures on alpha-tocopherol therapy alone. However, our successfully treated patients have also been numerous, including some on whom sympathectomies had been previously unsuccessful. This last, of course, is a parallel experience to that achieved in the treatment of Buerger's Disease.

Very recently, on the suggestion of Dr. Evan Shute, we have been trying a combination of oestrogen and alpha-tocopherol in an effort to reduce blood pressures in patients unresponsive to alpha-tocopherol treatment alone. Our results so far appear to indicate that this combination may hold a further clue to the successful management of this disease.

Theoretically this is a reasonable procedure, although we still insist that vitamin E is anti-oestrogenic.¹ All our evidence indicates that alpha-tocopherol dilates capillaries. One would therefore expect it to lower many or most elevated blood pressures. Why does it not do so at once? Because, presumably many persons have spasm higher in the vascular tree, perhaps at arteriolar level. There is evidence² that oestrogens act, in eclampsia for example, as arteriolar dilators. Hence, oestrogens used concomitantly with alpha-tocopherol might decrease the peripheral resistance in hypertensive patients not helped by a capillary dilator only.

This theory may help to explain why clinically identical cases at the menopause, such as those having vulvar pruritus³ or hot flushes,⁴ react to oestrogens in some cases and to alpha-tocopherol in others. This is true also of the late pregnancy toxæmias.⁵ Indeed, it is often possible to change one type of late toxæmia to the other, and back again.⁶ Such a feat is readily understandable if the principal effector organs in both instances are portions of the vascular tree anatomically very closely approximated and physiologically not too dissimilar.

Appended are some typical cases to illustrate each type of response mentioned above.*

(1) Mr. O. G.—age 65—first seen June, 1946—railroad engineer in Northern Ontario, pensioned because of hypertensive heart disease and a coronary thrombosis in January, 1946 (anterior myocardial infarction). His complaint was angina pectoris on exertion, with extreme weakness. On examination, his blood pressure was 220/120 and his pulse 60. His electrocardiogram was characteristic of anterior myocardial infarction. He was given a daily dose of 300 gm. of alpha-tocopherol. In October—four months later—he walked 8 miles into the bush, climbed a 250-foot observation tower, and, after talking for 20 minutes, walked 8 miles home. He later shot a deer three miles in the bush and dragged it out by himself. He was observed several times after that. His electrocardiogram changed steadily, with improvement even as late as November, 1947. On each occasion his blood pressure reading was 200 to 220 systolic, and 120 diastolic. He is completely asymptomatic and leads a normal life. For example, he cuts and chops his own wood.

* Blood pressures in all cases were taken to the fourth phase.

(2) Mr. G. D.—age 70. When first seen on May 12, 1947, the chief complaints were angina, dyspnea, and palpitation on exertion. In October, 1946, he may have had a coronary thrombosis, although an electrocardiogram taken at the beginning of treatment showed no evidence of either old or recent infarction. At that time, his blood pressure was 260/120 and pulse 60. He was given 100 mg. of alpha-tocopherol for three weeks, then 150 mg. for another two weeks. On June 13, 1947, his blood pressure was 194/100, pulse 64, and his angina gone, but dyspnea on exertion unchanged. The dose was increased to 200 mg. daily. On July 3, 1947, blood pressure 160/80, pulse 70 using the same dose. On August 5, 1947, his blood pressure was 160/75 and pulse 66, with no dyspnea, angina, or palpitation. At this stage he went back to light work. On December 27, 1947, his blood pressure was 130/70 and pulse 68; he had no symptoms and was working at a tannery, hanging up hides! He has remained well and active since.

(3) Mr. F. C.—age 57. He had had a coronary thrombosis with right bundle branch block. His chief complaints were angina, dyspnea, and weakness on exertion when first seen on May 20, 1947, with a blood pressure of 200/70 and pulse of 32. He was given 300 mg. of alpha-tocopherol daily. On June 16, 1947, his blood pressure was 180/70 and pulse 34. On August 26, 1947, he was symptom-free, his blood pressure 142/65 and pulse 36. He has been well and active since, working as a fireman.

(4) Mrs. D. L. C.—age 48. First seen on March 9, 1948. She had had a bilateral Smithwick in May and July, 1947 with no result. She was then "abandoned by her cardiologist." The blood pressure when she reported was 235/150 and pulse 68. She was given 100 mg. of alpha-tocopherol daily for five weeks. On April 21, 1948, her blood pressure was 180/140, pulse 80. The dose was increased to 200 mg. a day. On September 23, 1948, her blood pressure was 165/100, pulse 92. Currently she takes 250 mg. a day. She has not been seen lately but gives lectures, public demonstrations, and consultations on home decorating at a large department store.

(5) Mr. M.—age 69. Seen April 16, 1948. His blood pressure then was 155/70 and pulse 64. He complained of angina pectoris on exertion and dyspnea on mild exertion. He was given 200 mg. a day of alpha-tocopherol. On May 28, 1948, his blood pressure was 135/75 and pulse 72 and he had some angina. The dose was increased to 250 mg. On July 9, 1948, his blood pressure was 100/60 and pulse 76. The dose was then increased to 300 mg. per day and he has remained asymptomatic. He is an active farmer.

(6) Mrs. L. B.—age 53. She was seen first on March 1, 1949, with a blood pressure of 170/100 and neuritis. She was given 90 mg. of alpha-tocopherol. In two weeks her blood pressure was 180/100. Accordingly she was given 120 mg. of alpha-tocopherol and 5 mg. of stilbestrol per day. In seven days, her pressure was 156/88. Her alpha-tocopherol intake was promptly increased to 225 mg. per day, the stilbestrol remaining the same.

By April 5th, her blood pressure had fallen to 140/88 but she had some "vaginal spotting" that day. She was maintained on a smaller dose of the oestrogen.

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NOTES ON THE USE OF ALPHA-TOCOPHEROL IN THE MANAGEMENT OF ACUTE AND SUBACUTE VASCULAR OBSTRUCTIONS, AS WELL AS IN BURNS

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We have repeatedly^{1, 2, 3} called attention to the value of alpha-tocopherol in the treatment of acute and subacute vascular obstructions.

Results achieved in the management of acute and subacute venous obstructions are described in a series of consecutive, unselected cases in TABLE 1.

It is evident that alpha-tocopherol in large doses (200-600 mg. per day) has a marked effect in promoting the resolution of these acute venous obstructions and any attendant inflammatory reactions. Indeed, such cases respond so promptly and regularly to a proper dose, that, if there is any delay at all in their relief, one need only raise the level of dosage, often to 400-600 mg. per day. The results achieved in our earlier cases would undoubtedly have been improved had this been realized sooner. The dose is maintained for at least three weeks after clinical cure, but at a level rarely above 200 mg. per day. The platelet count is the best prognostic feature we have found. If this is low, the thrombus should respond readily. The converse is as true. Our theory concerning this has been given elsewhere.² We have had only one embolus in our series, and we doubt if this is more than a theoretical danger.

A typical acute or subacute arterial type of obstruction is *thromboangiitis obliterans*, a condition often accompanied by phlebitis, as is well known. In TABLE 2 is a tabulation of a small consecutive, unselected series of these cases. Of the sixteen cases, 10 and 15 constitute ugly controls. Of the other fourteen, only case 2 and 8, recent ones, have not been helped. Five cases have been followed for a year or longer. All five were helped.

While it is too early to assess the long-term results of our therapy of this notoriously difficult and chronic disease, it is obvious, at least, that alpha-tocopherol has as much immediate help to offer as rival surgical methods and is infinitely safer. Indeed, we have repeatedly been called on to treat cases very soon after surgical measures have revealed their inadequacy in the best hands. These patients have usually been so disillusioned when seen by us that it has been very difficult to secure their co-operation, particularly at the high and expensive level of dosage often demanded. However, we have, of late, been able to have such patients either meet their predecessors on tocopherol therapy or see colour photographs of our results, and by this means have achieved some degree of control over them until their improvement is undeniable and they can be induced to continue indefinitely. Dosage must often be large, and, needless to say, a reliable alpha-tocopherol must be used.

Arteriosclerotic Lesions. The same precautions in hypertensive arteriosclerotics must be taken as in all hypertensives. W. E. Shute has long

TABLE 1

	Sex	Age	Location	Type	Blood pressure	Treatment with α -tocopherol	Duration*	Subsequent
(1)	F	38	Rt. groin	6 days. P.P.†	124/74	400 mg.	5 days	0 in 2 yrs.
(2)	F	41	Rt. calf	6 days P.P.	162/68	400 mg.	3 days	0 in 2 yrs.
(3)	F	27	Rt. thigh	11 wks. pregnant	160/56	200 mg.	2 wks.	No recurrence at delivery or 1 yr. since.
(4)	F	40	L. knee	—	164/112	300 mg.	5 days	No recurrence 1 yr.
(5)	F	33	R. calf	12 days P.P.	120/70	300 mg.	4 days	"
(6)	F	32	L. calf	3 mos. pregnant	120/70	450 mg.	2 wks.	Cut dose to 150 mg. a day. Recurred 5 wks. later.
(7)	"	"	"	4½ mos. pregnant	130/80	750 mg.	10 days	Much improvement in 4 days.
(8)	F	30	R. calf	2 days P.P.	116/70	300 mg.	8 days	No recurrence 8 mos.
(9)	F	21	R. thigh	2 days P.P.	120/60	400 mg.	3 days	No recurrence 8 mos.
	M	34	R. thigh	Varicose	134/76	500 mg.	2 wks.	Much tobacco. Recurred in 6 wks.
	—	—	"	—	—	750 mg.	10 days	No recurrence for 6 mos. Stopped tobacco.
(10)	F	27	L. thigh & calf	8 mos. pregnant	90/64	400 mg.	10 days	No recurrence at delivery.
(11)	—	28	Rt. leg	8½ mos. pregnant	110/70	375 mg.	14 days	Was taking 240 mg. a day when it happened. No recurrence at delivery or since.
(12)	F	54	2 legs	In bed with coronary thrombosis 5 wks. before	110/70	150 mg.	5 wks.	Steady improvement.
(13)	F	53	L. leg	In hospital for colitis 8 wks. before	128/70	400 mg.	7 wks.	Recurred after 2 mos. when on 150 mg.
(14)	—	—	"	—	—	400 mg.	10 days	Well since.
(15)	F	30	L. Calf	3 mos. pregnant	116/80	300 mg.	12 days	In 7 days could walk a block.
	F	52	Embolus rt. thigh 5 mos. before. Can walk 2-3 minutes. Fibrillating.	Post-pneumonic	130/100	150 mg.	5 mos.	In 3 mos. could walk 1 block; in 5 mos., 2 blocks.

* Duration—period required for clinical cure.

† P.P.—post-partum.

TABLE 2

Case	Sex	Age	Tobacco	Site	Duration	B.P.	Diagnosed	Previous Therapy	Exercise Tolerance	3-4 Wks.	2 Mos.	8 Mos.	12 Mos.
1	M	47	2+	R. ft.	8 mos.	190/128	Toronto Hospital	Symp. advised	$\frac{1}{2}$ block	170/116. Walks 6 blocks. Smokes	Back at work. Steady gain		
2	M	46	2+	L. ft.	8 yrs.	188/116	Toronto Hospital	L. symp. amp. advised Gangrene L. gt. toe	Crutches. Sleeps poorly	Pain severe	Necrotic dist. phal. amp.		
3	M	48	3+	Both ft.	4 yrs.	?	D.V.A. Hospital	L. leg. amp. & other leg advised amp. Ulcer on stump	Crutches. Pain	No pain. Ulcer healed. Wears prosthesis	Off E ulcer recurred. Healed quickly on E		
4	M	43	4+	Both legs	8 yrs.	?	N. Y. & Toronto	Boot. salines	1 $\frac{1}{2}$ blocks. Much pain	L. phlebitis. Less pain. Toe ulcers healed	$\frac{1}{4}$ mile. Some pain at night	Smokes. Stopped E & recurred. Reduced E & rt. ft. pain. P.t. on rt.	
5	M	38	4+	L. ft. & leg	3 yrs.	108/72	R.C.A.F. biopsy	L Symp. (1947). Toe Amp. (1947)	4 blocks	$\frac{1}{4}$ mile. 9 holes golf	Ulcer healed. No pain	2 miles. Ran 100 yards. Smokes	
6	M	50	0	L. ft. & leg	14 yrs.	175/90	Minn. Clinic	L. Symp. Recurrent ulcer toe. Finger ulcers healed by symp.	Walks 4 blocks	Much less pain. No claud.		Walks 1 mile	
7	M	56	?	Both legs Rt. worse	1 yr.	180/120	London Hospital	Symp. advised. Gangrenous ulcers rt. foot			150/80. At work. Ulcers healed	Died suddenly (coronary or embolus)	
8	M	35	0	Both ft.	9 yrs.	124/88	Montreal Hospital	Bilateral symp. (1946)	Ache to hips		No help	Losing numbness. Pain same	

9	M	42	2+	Both ft. Phlebitis 1 ft.	9 yrs.	140/80	Toronto Hospital	Boot. Typhoid	Presser. Stopped q. 40 mins. to rest & massage legs		Less pain	Steady improvement	Works 10 hrs. free of pain. Stopped E & pain recurred
10	M	41		Both ft.	15 yrs.	110/70	Toronto Hospital	Ft. Symp. (1932). Rt. toes amp. (1933). L. Symp. (1940). L. Toes amp. (1940) Symp. advised	Can't sleep		No pain. Rt. toes ulcerated. Stopped E for general pruritus	R. leg amp. Stump unhealed	
11	M	36	2+	Both ft. Discoloured great toes	7 yrs.	120/80	Toronto Hospital	Symp. advised	$\frac{1}{2}$ block		Pain if stands 5 mins.		No smoke. Much helped
12	M	47	+	Both legs	8 yrs.	?	Minn. Hospital	Amp. leg (1941) for pop. embolus	"Walks short distances"				Stands longer. Climbs stairs. Forearm thrombosis transient
13	M	69	2+	Both legs	5 yrs.	264/130	Toronto & Cleveland Hospital	Rt. leg amp. Lt. symp. Ulcer R. gt. toe	100 yards		Ulcer healed		
14	M	48	3+	Both' legs (self-treated)	3 yrs.	?	Toronto Hospital	Symp. advised 12 prs. shoes (sizes 8-12). Nails short or heaped up.	$\frac{1}{2}$ block		Much help so stopped E	Off E & severe pain	Smokes. Walks 2 miles. Nod. p. nor p.t. Nails normal.
15	M	23	3+	L. leg	2 yrs.	130/80	London Hospital	Symp. advised. L. ft. gangrene	Crutches. No sleep. Much pain		Amp. above knee		
16	M	39	3+	Both legs	2 yrs.	126/76	Edmonton Hospital	Salines. Advised amp.	Phlebitis. L. foot white. Unable to walk				Bicycles to work Little pain. Walks $\frac{1}{4}$ mile. Teaches boxing

insisted¹ that the initial dosage in such patients *must* be small and can be increased only gradually. Indeed, the occasional patient quickly comes to a dosage limit beyond which his blood pressure does not permit him to go. This is one of the major precautions in Vitamin E therapy.

The application of these experiences to analogous lesions in the coronary vessels is obvious. And so we leave our cardiological critics impaled on the tines of this fork:

(a) If alpha-tocopherol brings about vascular dilatation and resolution of thrombi in the femoral branches of the aorta, why cannot identical changes occur in the coronary branches of the same aorta 12 inches away? Indeed, there is a profound teleological reason why coronary and leg vessels should react in close parallel.

(b) If alpha-tocopherol induces a better blood supply in the coronary system but fails to relieve cardiac pain our critics must bring forward a new nonischaemic theory of the origin of such pain!

We have always been interested in Steinberg's wonderful studies⁴ on fibrositis and scars. He demonstrated that old scars often relaxed when the patient was given high-dosage tocopherol. The tocopherol ointment applied locally in one of our burn cases* always provoked visible capillary dilatation, until sometimes, in the larger wounds, the distended capillaries in the bed of granulation tissue actually sagged by gravity. Stopping the tocopherol ointment and applying at once another medication in the same ointment base allowed the "button wounds" to flatten out; applying tocopherol ointment heaped them up again. This could be repeated indefinitely. Healing was rapid, moreover. Furthermore the resultant scars were unusually flat, pliable, shallow, and broad. These scars did not contract after healing, as scars usually do. To avoid such scar contracture in burns is the reason *par excellence* for skin grafting. The implication of these studies for the healing of fresh wounds and burns is challenging.

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Discussion of the Papers

DOCTOR H. I. LIPPMANN (*Adjunct Attending Physician, Peripheral Vascular Dept. Montefiore Hospital, Bronx, N. Y.*): In the following, I wish to report on the treatment of arteriosclerotic peripheral vascular disease with alpha-tocopherol acetate,† as experienced in the peripheral vascular wards of Montefiore Hospital, Bronx (Chief: Dr. Samuel Silbert). The study was made following the remarkable claims advanced by Shute and collaborators, in January, 1948.

It is a truism that the evaluation of any drug in the treatment of peripheral

* Color photos of burn cases were shown at this point when author presented his paper.

† Eplynal acetate was used and furnished in part by Hoffmann-La Roche, Inc. Nutley 10, N. J., Medical Dept.

vascular disease is difficult. It has to be made against the background of the natural course of the disease, so that spontaneous improvements will not be erroneously attributed to any one drug. Dr. Shute's data on a phlebitic and a diabetic ulcer in the process of healing while vitamin E is being administered, reveal, I believe, merely the good results of conservative treatment. Similar, and occasionally even better results, can be observed in untreated cases, as we have learned in our clinics at Mount Sinai and Montefiore Hospitals, and in the wards of Montefiore Hospital, where some of our patients have been followed for many months under conservative treatment.

When the present study was initiated, it was felt that a well-controlled small group of patients on no other medication than vitamin E or placebos was preferable to a large number of ambulatory patients, as far as the evaluation of the drug was concerned. Six patients with arteriosclerotic peripheral vascular disease were selected for the study, 3 women and 3 men, 5 diabetics and 1 non-diabetic, their ages ranging from 48 to 74 years of age. The following criteria were observed:

(1) An observation time at the hospital without any treatment for at least 4 weeks preceded the administration of the drug.

(2) The disease was not detectably progressive in any one case selected. Only slowly developing lesions which had shown no previous healing tendency were included, but no spreading ulcers or gangrene.

(3) In our treated patients, not more than 3 toes (and in one case the side of the foot) were involved.

(4) Body weight, caloric and protein intake, and tolerance to carbohydrates had been constant prior to and during medication. All patients were moving about in rolling chairs and were not kept on or changed to complete bed rest prior to or during the period of medication with vitamin E.

Alpha-tocopherol acetate was given by mouth at 100 to 200 mg. tid. (300 to 600 mg. daily) for a period of 2 to 4 months. In one case only, it had to be discontinued after one week. No untoward reactions were encountered in any case. No other medication, with the exception of necessary sedation, insulin, or digitalis was given. Six patients were observed without vitamin E medication as a control group. The results reveal:

(A) Organic vascular occlusion and the formation of collateral circulation are uninfluenced by alpha-tocopherol. This was proven by repeated examination of the patency of pulses, oscillometric and skin temperature readings, before and after posterior tibial nerve block with procain, reflex heat (Landis-Gibbon), reactive hyperemia (Lewis-Pickering), or the flush produced by injection of papaverine into the femoral artery, whenever possible.

(B) The pain due to ulcer or gangrene was uninfluenced by vitamin E. In both treated and untreated groups, 2 patients experienced a marked diminution of pain.

(C) No favorable influence upon the healing of ulcers by vitamin E could be observed. In the treated group, one ulcer healed; in 4 patients, the gangrene initially involving one, two, or three toes spread to more toes or

the foot. An amputation at midleg level had to be made in one of these patients during the period of medication. In one more patient, the ulcer developed into an abscess and cellulitis for which incision and drainage had to be made in the usual fashion. In the control group, 3 patients healed spontaneously.

To study the problem of ulcer healing further, two young women were added to the group. Both were known to us for years, and both suffered from extensive lower extremity ulcerations associated with ulcerative colitis. No etiological infectious agent had been found in the leg ulcers. Since previous plasma infusion had given the relatively best therapeutic results, the etiology of an undetermined deficiency had been postulated and a trial with vitamin E appeared to be indicated. The drug was administered at 200 mg. tid (600 mg. daily) over a period of 4 months without any benefit.

I would like to add that, in our series, insulin requirements did not change after administration of vitamin E, except in the one patient whose leg was amputated. Following the operation, insulin could be discontinued. There is no reason to assume that any other cause than removal of a gangrenous member was instrumental in improving the tolerance to carbohydrates in this patient.

In conclusion, none of the claims of Shute and collaborators was confirmed in our controlled small group of hospital patients with arteriosclerotic peripheral vascular disease, treated with alpha-tocopherol. In our hands, the drug has been ineffectual, and no indication could be found for the use of alpha-tocopherol in the treatment of arteriosclerotic peripheral vascular disease.

DOCTOR A. VOGELSANG (*London, Ontario, Canada*): I have never seen any harmful effects from the large scale administration of vitamin E to patients with hypertension or with rheumatic heart disease. I believe that their warning in regard to possible harmful effects in such patients is unnecessary. I have had twelve cases of Buerger's disease which have responded to alpha-tocopherol therapy alone. In nine of these cases, there were no initial oscillometric pulsations in either leg. One patient had a palpable popliteal pulsation in one leg and the remaining two had no palpable pulsations at all in the lower extremities. I cannot account for the failure of vitamin E therapy in the cases reported by Dr. Lippman.

DOCTOR GEORGE C. DOWD (*Boston Evening Clinic and Hospital, Boston, Massachusetts*): It appears that we are at the crossroads of confusion. We have on the one hand several reports which completely deny the therapeutic effects of alpha-tocopherol; on the other hand, we have reports by equally enthusiastic investigators which attest to the efficacy of this preparation. What this investigator proposes to do, is present his concepts and experience. Over the last three years, this author has seen some three hundred cases of arteriosclerotic cardiovascular disease at the Boston Evening Clinic and Hospital and in his private practice. Of these, some 25 per cent were hypertensives. Each patient was given a thorough diagnostic work-up and tocopherol therapy.

Before we can discuss intelligently the management of these problems, we must refer, for a moment, to the degenerative changes associated with the aging process. Associated with aging, there appears to be a generalized "brown atrophy" of liver, heart, and kidney, with concomitant lipid infiltration as a precursor of sclerosis. Also, one sees hyalinization of connective tissue and atheromatosis of the vascular tree. As a result, one is confronted with a progressive ubiquitous ischemia and attendant decrease in organ function.

With the above concept in mind, we have attempted to treat the various cardiovascular problems by treating any associated organ defects simultaneously with the cardiovascular problems. To that end, we have not hesitated to use high potency vitamin B Complex, methionine, parenteral liver extract, and other agents synergistically along with alpha-tocopherol. The latter product was added after the maximal effects of the synergists had been produced.

In our series of arteriosclerotics, some 25 per cent were overweight; the balance were cachectic. Each patient was studied as objectively as possible: careful physical examination, laboratory studies, electrocardiographic, and fluoroscopic studies were made; and a special sound quantitator was used in evaluating heart sounds. Our results were as follows:

In the hypertensive group of patients, some 75 per cent had a blood pressure drop of 10-40 mm. systolic/10-30 diastolic pressure. Fluoroscopically, there was no significant change; electrocardiographically, there were slight changes in the QRS complex, with a tendency to decrease in the number of fibrillations in those who were fibrillating. There was also a tendency to higher amplitude in the QRS complex. The most striking response appeared in the greater excursions of the decibel indicator on the sound quantitator. Blood urea nitrogen and cholesterols became more nearly normal, and kidney function tests were improved in those patients who had concomitant renal pathology.

In the peripheral vascular group, we have seen eleven cases who have been studied by the usual methods. Seven of these were arteriosclerotic toe ulcers. Two were gangrene cases, and two had varicose ulcers. On tocopherol and synergistic adjuvant therapy, six cases healed. Four cases are improving slowly, and one case has failed to respond to treatment. The two gangrene cases have cleared up.

Now a word of criticism. The favorable group of investigators, who are very sincere and industrious Canadian colleagues, have had very extensive clinical experience with alpha-tocopherol in the management of cardiovascular disease. The studies have been carried out at London, Ontario, a community where they have not had the opportunity for exacting clinical research, such as one finds in a large urban hospital. On the other hand, the opposite group, equally sincere and equally industrious, have carried out very small series of accurate studies. The criticism, to be leveled at the latter group, is that: (1) in several instances, the product used has been of insufficient dosage and potency; (2) the patient was suddenly withdrawn from conventional therapy and placed on "E" alone (this is not the technique

followed by either the Canadian group or ourselves); (3) the series presented is too small. On the basis of our own independent studies over three years, it is felt that the final answer, in reference to the efficacy of alpha-tocopherol in cardiovascular disease, has not been determined. Both proponent and opponent groups should not be so positive in their attitudes. What is needed, and very acutely, is a long range study on large numbers of patients, over several years, to determine whatever merits may be present.

DOCTOR E. V. SHURE: Biologists here must be amazed to find how different man is from all the experimental animals. Despite all the evidence that he has lived for years past on a diet deficient in tocopherols, he develops no tocopherol deficiency! Like other species, if tocopherol deficiency could develop, it might involve nearly any system. But the cardiovascular system is forbidden ground! There it *must* not play any rôle! All this used to amaze us, too, of course.

It is obvious that such wide disagreement as has been here demonstrated will scarcely permit the issues at stake to be resolved in this monograph. And I have no intention of trying to answer our critics point by point.

I could say, in general, that they are still writhing on the tines of the fork because of the dilemma presented to them in our paper, and long may it disturb them. If the evidence we have presented has been disbelieved, we in our turn, have had the same experience with the findings of the Cornell group and those just mentioned by the local discussants of our paper. The contradiction of our observations, which have been very numerous and made over a period of years, has been just a little too complete. Burgess and Pritchard, Stritzler, Pennock, and ourselves could be partially mistaken, but are not apt to be utterly deluded.

Almost all the cases presented in our series were chronic, had been diagnosed in the best clinics and hospitals, had had every conceivable known treatment before falling into our hands, and were stabilized in failure. It is absurd beyond measure to say that what was achieved after they received tocopherol therapy would have happened in every case in any event. That argument wears too thin after a time. To say that our diabetic's change in insulin tolerance was to be expected, for example, ignores the fact that he had been stabilized for 6 years previously on his dosage. Some better type of objection is long overdue.

May I conclude by reading into the record the story of the low-sodium diet. It is now highly regarded generally, especially since a Boston group revived it in 1946. But how many of us know its history? It dates back to a paper prepared by Edwin Wheeler, William Bridges, and Paul White, and presented at the A.M.A. Meeting in 1946, although it appeared in the J.A.M.A., Volume 133, page 16, in 1947.

In the discussion, Dr. Fred M. Allen of New York made some indignant remarks. He pointed out that although he first published this diet in 1922, it had been condemned for the next 24 years. It had been condemned in two J.A.M.A. editorials, in symposia at the New York Academy of Medicine, and in "every review and book by every authority in this country." "During the 20 years that I was standing alone against all the professors in all the

Medical Schools of this country, I insisted that the issue was strictly one of accuracy of clinical observation. The final decision should note this point and also my demand for a retraction of editorial misstatements." How could the low sodium diet be so useless for 24 years and so valuable since 1946? The answer is incredible.

DOCTOR W. E. SHUTE: The value of alpha-tocopherol in heart disease and peripheral vascular disease is now known at first hand by about 3,000 doctors in Canada alone, but its vicissitudes at the hands of some of the senior specialists remind us of other events in medical history. You all know, for example, what would have happened if a physician with no experience with insulin had been given all the insulin he wanted and one hundred diabetic patients 26 years ago, and had then proceeded to treat them, to the best of his abilities, with just the information that some group in Canada had said that insulin was useful in this disease. This hypothetical doctor proceeded to give them all a set dose, let us say 50 units a day. You all know what must have happened! In his case, too, his blind controls would have fared better than his blindly treated patients—for the obvious reason that any good medication is potent and therefore dangerous. He would have achieved significant results in only a very few, and many would have been made worse. Would his experience prove that insulin was of no value in the treatment of diabetes mellitus? Suppose that his supply of insulin consisted of 100 different makes whose contents and labels bore the relationship to each other characteristic of many of the 100 or more brands of Vitamin E currently on the market—as they say in the movies: "Any resemblance between the two is purely co-incidental." What would be his success with this new treatment of his 100 cases of diabetes mellitus?

Our critics are fond of saying that the results achieved by us would have developed anyhow—and this in spite of our insistence that our patients had in almost every case received the best-known treatment for as long as 10 to 20 years previously. These remarks merely convince us that our critics should no longer continue practising medicine, since either (a) they and their peers must have delayed those "spontaneous cures" from appearing until they relinquished the cases to us, or (b) they must long have been receiving fees to which Mother Nature had prior right.

We are fascinated by the workings of a mind that can claim that gangrenous areas in the lower extremity associated with calcification in the posterior tibial artery are not due to basic and extensive vascular pathology.

It is apparent that the material our discussants prepared and have read in rebuttal had little bearing on the actual text and content of our presentation. We merely regret that they seem to have been quite unprepared for our studies on the healing of burns. Surely, the speed with which our results were achieved should have been convincing.

TOCOPHEROL THERAPY IN STASIS ULCER AND STASIS DERMATITIS

By Conrad Stritzler*

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Early in 1948, Shute and his colleagues¹ reported improvement in leg ulcers in patients on tocopherol therapy for cardiac disorders. Soon after, Burgess and Pritchard² reported healing of a nodulo-ulcerative granuloma of the legs with tocopherols, and the same authors³ noted improvement in sclerotic ulcers of the legs with vitamin E.

This study represents an effort to confirm Shute's work as it applied to stasis ulcer. Further it was felt that if tocopherols† are effective in stasis ulcer, they may also be effective in a pre-ulcer state, stasis dermatitis.

Thirteen patients with stasis ulcer and fourteen patients with stasis dermatitis were chosen for this study. All had failed to respond to orthodox measures given over a period of months to years. These measures included various topical remedies, occlusive dressings, local pressure, silver or aluminum foil, and skin grafts. Most of them were kept under observation for three to four weeks on bland local therapy in order to gauge personally the degree of improvement, if any, under such treatment. A few cases had been under competent supervision without improvement for so long that treatment was begun without this preliminary period of observation. All were treated on an ambulatory basis with no restriction of their activities. Those patients who showed any significant degree of improvement on topical therapy were not included in this study.

Clinical Data

There were four females and twenty-three males, three colored, the rest white. Ages varied from twenty-five to seventy-seven, and duration of symptoms ranged from three months to more than forty years. Serologic tests, urinalyses, and hemograms were normal. It was felt that the condition was due to chronic venous insufficiency, resulting from varicose veins, chronic thrombophlebitis, or both, in all cases.

Patients with stasis dermatitis showed the usual patchy or confluent involvement of the legs from knees to ankles. Ulcers varied in size and number, from a single dime-sized lesion near the internal malleolus to large, confluent ulcers encircling the lower third of the leg.

Treatment consisted of the oral administration of capsules of 400 mg. of mixed tocopherols (equivalent to 100 mg. of dl, alpha-tocopherol) three times daily—a half hour before meals, or midway between meals. Mineral oil by mouth was avoided, and patients were asked to avoid excessive fat

* We wish to express our gratitude to Dr. Harvey L. Myers for permission to treat several patients from the Peripheral Vascular Disease Clinic, Queens General Hospital, Jamaica, N. Y.

† U. S. Vitamin Corporation supplied the preparations of vitamin E (capsules E Toplex and ampules of water soluble E).

intake. No adjuvant therapy was given. If after six weeks of oral therapy there was no significant improvement, they were given, in addition, intramuscular injections of a solution of 50 mg. of dl, alpha-tocopherol in 2 cc. of water, to which had been added sorbitan monolaurate⁴ as a solubilizer, 0.5 per cent chlorbutanol and 2 per cent procaine to control local pain. Without the procaine, this preparation proved extremely irritating locally. With 2 per cent procaine there was no immediate pain, but a mild, localized ache occurred several hours after the injection, lasted only a short time, and did not incapacitate the patient. Injections were given two or three times weekly, depending on available clinic facilities and the ability of the patient to report regularly.

It was soon noted that all patients with stasis ulcer and most patients with stasis dermatitis showed little improvement until parenteral therapy was instituted. Only four patients with stasis dermatitis have shown significant improvement on oral therapy alone. Improvement was generally not manifest until therapy had been given for six weeks or longer orally, plus an additional two weeks of conjoint oral and parenteral therapy. Of the thirteen patients with ulcer, at the time of this report, one patient has healed completely; one shows striking improvement, with fifty to seventy-five per cent healing; three were moderately improved, with twenty-five to fifty per cent healing; five showed slight but significant improvement with ten to twenty-five per cent healing; and three showed no improvement at all. Thus, ten of the thirteen patients with ulcer showed improvement varying from ten per cent to complete healing after four months of treatment. Smaller ulcers, and those of more recent onset, responded best. Several foul smelling ulcers with necrotic bases soon became clean and granulating. The smaller ulcers crusted over, and healing progressed beneath the crust. The defect seemed to fill in both from the base toward the surface and from the edges centripetally. There was little effect on pain in one patient who complained of severe pain at the ulcer site, but, in a second patient, pain gradually subsided with occasional exacerbations.

Of the fourteen patients with stasis dermatitis, two cleared completely, and the rest showed fifty to seventy-five per cent improvement following two to four months of therapy. Repeatedly, they relapsed when treatment was discontinued for two weeks or more and improved again on resumption of therapy. Maintenance doses of 200 mg. to 300 mg. daily by mouth were necessary to maintain improvement. In two patients with superimposed pyoderma, there was no improvement until the pyoderma had cleared up.

There were no reactions in patients while on oral therapy alone. Reactions occurred in three patients while on combined oral and parenteral therapy. One developed a mild generalized pruritis with no objective cutaneous manifestations after the fourth injection. This responded promptly to antihistaminics orally and did not interfere with further treatment. It recurred irregularly and could not be reproduced with any degree of precision by either oral medication, injections of procaine, chlorbutanol, alpha-tocopherol in sesame oil, or the aqueous solution of tocopherol with sorbitan monolaurate complex. The second patient developed a mild gen-

eralized urticaria after almost three months of combined oral and parenteral treatment. This cleared a few days after therapy was discontinued and could be reproduced regularly only when a synthetic alpha-tocopherol preparation was given orally. It was necessary to discontinue oral and parenteral therapy, but a 5 per cent tocopherol ointment in carbowax locally at the ulcer site could be tolerated. This patient is considered to have a proven urticarial sensitivity to tocopherols. The third patient developed localized hemorrhage, swelling, and tenderness after the eighteenth injection, with a sustained pyrexia of 104° F. Previously injected sites also became hemorrhagic, edematous, and tender. The fever responded in three days to antihistamine therapy, but the tender nodules persisted for several weeks. It was considered a form of acquired localized sensitivity analogous to the Arthus phenomenon. This patient later tolerated tocopherols orally, and injections of chlorbutanol, procaine, and alpha-tocopherol in sesame oil were also well tolerated. It is possible that the reaction was due to the solubilizer. The severity of the reaction made it difficult to try the effect of reinjecting the preparation which presumably was responsible for the reaction.

Pre- and post-treatment biopsies were obtained in three patients with stasis ulcer. The pre-treatment biopsies show the usual pseudoepitheliomatous hyperplasia of the epiderm at the margin of the ulcer, granulation tissue invading the corium, round cell infiltration, and deposits of hemosiderin. The post-treatment biopsies were taken from a site immediately adjacent to the previous one, after almost four months of treatment with moderate improvement. They show changes in the direction of normal, but nothing which can be interpreted as indicating a tendency to the formation of new capillaries or any detectable effect on the collagenous substance of the corium.

Comment

Shute¹ suggested that the effect of tocopherols on leg ulcers may be due to the reopening of closed capillaries or the proliferation of new capillaries. He postulated the latter as the more plausible explanation, since there was a latent period of a month or more before improvement became evident. While this seems logical, and we were able to confirm the lag of several weeks before improvement occurs, we were unable to confirm this opinion by our biopsy studies. Studies with fluorescein⁵ are contemplated in the hope that they may shed light on this problem.

The possibility that tocopherols exert their effect on tissue enzyme systems may be considered. In view of the relatively large doses and prolonged administration necessary to produce clinical effects, it is possible that these effects are due to the pharmacodynamic action of the tocopherols rather than to any vitamin effect. Hickman,⁶ however, thinks that this indicates a severe tocopherol deficiency which requires prolonged therapy to correct.

One other point is worthy of comment. Among our patients with stasis dermatitis complicated by pyoderma, there was no effect from tocopherol therapy until the pyoderma had cleared up. Infection evidently interferes with the action of tocopherols on stasis dermatitis. Infected, necrotic ulcers,

however, became clean and granulated with no therapy other than tocopherols orally and parenterally. We have no explanation to offer for this discrepancy.

Will tocopherols prove useful in preventing stasis ulcer in patients with chronic venous insufficiency and stasis dermatitis? Will their effectiveness be more evident if used as an adjuvant to other and more orthodox measures, rather than as the sole method of therapy? Why is parenteral therapy necessary in most cases? Is absorption from the gastro-intestinal tract inadequate, or is there an optimum blood level which cannot be reached with oral therapy alone? Are larger doses indicated? Is alpha-tocopherol alone as effective as mixed tocopherols, or is beta-, gamma-, or delta-tocopherol equally or more important? These and many other questions remain to be answered.

Two patients with discoid lupus erythematosus and two patients with pruritis ani were treated, with results which suggest that further study of tocopherol therapy in these conditions is warranted. Burgess and Pritchard⁷ have reported improvement in discoid lupus erythematosus with this form of therapy. In one patient with stasis dermatitis a coincident pruritis ani improved. It was tried on a second patient with pruritis ani and menopausal symptoms, and both conditions improved. We do not offer tocopherols as a form of therapy for pruritus ani, but merely mention these findings as an interesting by-product of our study.

Conclusion

From this preliminary study, the conclusion is justified that adequate tocopherol therapy may be of value in most cases of stasis dermatitis and in some cases of stasis ulcer. A final evaluation cannot be made until more cases are studied and our patients are followed for a longer period of time. Study of this problem is being continued and amplified.

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Discussion of the Paper

DOCTOR M. L. QUAIFFE (*Research Laboratories, Distillation Products, Inc., Rochester, N. Y.*): That vitamin E dissolved in oil, such as sesame oil, effects no increase in blood vitamin E levels following intramuscular injection

has been reported by several investigators. We have confirmed this with rats. Intramuscular injection of as much as 8 mg. of α -tocopherol in olive oil caused no rise in the blood tocopherol levels of 200 gm. vitamin E-deficient rats within 24 hours after injection. On autopsy, a pocket of undissolved oil was found at the injection site.

Conversely, when the same quantity of α -tocopherol was dissolved in Tween 80, intramuscular injection of it caused an increase in blood serum vitamin E from an initial level of 0.1 up to 4.0 mg./100 ml. at 24 hours. (Intramuscular injection of Tween 80, itself, caused no increase in blood vitamin E). The injected muscles appeared normal on gross inspection.

Intramuscular injection of α -tocopherol dissolved in Tween 80 (2 mg. 0.1 ml.) was made on a series of vitamin E-deficient rats (approximately 200 gms. in weight). They were sacrificed at varying intervals, and blood vitamin E levels were determined. These are listed below.

<i>Blood serum tocopherol</i> (mg./100 ml.)	<i>Time after injection</i> (hours)
0.14	0
0.19	0
0.34	2
0.53	3
1.1	6
1.1	12
1.4	24
0.84	48

Evidently, α -tocopherol is readily absorbed into the blood stream following intramuscular injection when it is solubilized with Tween 80. We have made no injections of α -tocopherol dissolved in Tween 80 into humans because of lack of knowledge about the possible toxicity of Tween following intramuscular injection.

DOCTOR J. F. BURGESS (*Montreal General Hospital, Montreal, Canada*). My results in the treatment of leg ulcers paralleled Doctor Stritzler's findings fairly closely. On the other hand, however, I was unable to obtain much benefit in cases of pure eczematization of the legs. I was interested in the group of cases that I studied because of the associated sclerosis and thickening which was present and which was evidence of connective tissue disease. My series of cases of leg ulcer seemed to me to be in a quite different category from those presented by Dr. E. Shute. I feel that, in my cases, vascular stasis was an unimportant phase of the whole clinical picture. In one case, where clinically the sclerosis was most severe and was characterized histopathologically by a very marked, productive fibrosis, after healing of the ulcers had occurred, continuation of vitamin E therapy combined with crude wheat germ over a year resulted in a marked pliability plus increased elasticity and lessening of pigmentation. Possibly this may be attributed to continued therapy or to a subsidence of the whole pathological state. Unfortunately, we have not as yet been able to obtain further biopsy material.

DOCTOR LOUIS FREEDMAN, (*Director of Research, U. S. Vitamin Corporation New York, N. Y.*): During the discussion of Dr. Stritzler's paper, questions were asked concerning the composition of the product used by Dr. Stritzler

and the toxicity of the sorbitan monolaurate derivatives used as solubilizer in the product. The writer was called on by Dr. Stritzler to discuss these questions.

The product used by Dr. Stritzler was made up in our laboratories to contain 50 mg. of dl, alpha-tocopherol (synthetic) in 2 cc. of an aqueous solution. The solution contained, in addition to the dl, alpha-tocopherol, 26 per cent, weight to volume, of a polyoxyethylene derivative of sorbitan monolaurate (popularly known as "Tween 20") as solubilizer, with 2 per cent procaine hydrochloride, and .5 per cent chlorbutanol as a stabilizer.

The polyoxyethylene derivatives of sorbitan monolaurate are relatively nontoxic to man and nearly all other animal species, with the exception of the canine species. Krantz ("Pharmacodynamic Studies of Polyoxyalkylene Derivatives of Hexitol Anhydride Partial Fatty Acid Esters", J. Pharm. and Exp. Therapeutics, Vol. 93, No. 2, pp. 188-195, June, 1948 and in private reports) has shown that the "Tweens," in 5 and 10 per cent solutions, can be injected intramuscularly for as many as twenty successive days without any deleterious effects. All animals, other than the dog, appear to tolerate intramuscular injections well. And even intravenous injections in all animals, with the exception of the dog, appear to be tolerated without any toxic manifestations. In clinical trials of vitamin solutions containing 5 per cent of Tween 20, intramuscular injections in about 500 patients showed no untoward effects and good tolerance. No hemoglobinuria and no change in the blood pictures were reported by five different investigators (unpublished reports).

The three reactions reported by Dr. Stritzler in his series of cases may have been due to the higher percentage of "Tween" present in the product. These reactions may be considered either as direct allergic reactions or acquired allergy, either to the "Tween" or to the tocopherol, or even possibly to the procaine present in the product. Intolerance, with several hundred injections of the same product without procaine, was unreported.

Krantz has shown that the peculiar reaction noted in dogs with the "Tweens" is one of allergy, since the reaction may be prevented or ameliorated by prophylactic or palliative treatment, respectively, with anti-histamines. This protective action of anti-histamines has been confirmed by our own experiments, not yet published.

The peculiar susceptibility of the canine species to "Tweens" is not understood.

VITAMIN E IN RHEUMATIC DISEASES

By Morris Ant and Erwin Di Cyan

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This paper is based on the premise that a sample of 100 individuals may be representative of the analogous population. One hundred non-selected individuals with complaints referable to or suggestive of rheumatism might be expected to include the various forms of rheumatic diseases.

Specifically, it was found that 100 patients (90 per cent of whom were referred by physicians so that metabolic treatment might be instituted for their rheumatic complaints) included various forms of the rheumatitides. These comprised various degrees of duration, severity, and incapacity. The diagnoses included the everpresent rheumatoid arthritis, manifestations of osteoarthritis, and skeletal muscle disorders generally designated as muscular rheumatism. These were of traumatic, endocrinologic, infectious, and probably nutritional origin.

Our obvious intention was to relieve the basic complaints, and, since the interest of one of us (Ant) is primarily in the realm of clinical metabolism and nutrition, a particular attempt was made to treat the anomalies of metabolism and nutrition by dietotherapy, and particularly by the generous use of vitamin E intramuscularly, orally and topically. In 16 per cent of the patients, such treatment was of little or no avail. However, in about 84 per cent, a marked and appreciable improvement occurred, with a mitigation or cessation of attendant disability. It is recognized that results of this nature have been reported for many treatments, whether these were injections of gold or psychotherapy; and no doubt part of the number of improvements may be due to the agency of time and natural remission of the disease.

Rheumatic diseases are not essentially local, but systemic conditions, and these systemic conditions may either *initiate*, *precipitate*, or *sustain* a rheumatic entity. Most coexisting conditions present before treatment with vitamin E (TABLE 1) were still present after treatment with it. No particular attempt was made to treat the coexisting conditions, and only a small proportion of such coexisting conditions improved. This constituted part of our control.

Vitamin E (intramuscularly, orally, and topically) was employed on the theory that: (a) being the vitamin most broadly distributed and most plentifully present in the system, it would have, necessarily, broadly applicable effects; (b) symptoms of its deficiency would be protean because of its wide distribution; and (c) its usefulness in fibrositis having been favorably reported,^{1,2,3} its use in muscular rheumatism and similar rheumatic entities would not be unwarranted. Vitamin E could not reasonably be expected to constitute a specific in the treatment of rheumatic disease. At best, no more was expected than a measure of aid in the metabolic rehabilitation of the rheumatic patient.

Osteoarthritis is frequently metabolic, and muscle spasm does accompany

TABLE 1

GENERAL DISTRIBUTION OF NUMBER OF COEXISTING CONDITIONS IN 100 INDIVIDUALS TREATED FOR RHEUMATIC DISEASES WITH VITAMIN E

Anemia	2	Hyperthyroidism	8
Cardiovascular	4	Loss of weight and	
Cirrhosis of liver	2	general malnutrition	4
Constipation	2	Neuralgia	5
Diabetes	32	Neurosis	2
Gastro-Intestinal	6	Obesity	9
Genito-Urinary	10	Respiratory	4
Gynecological	2	Sensory changes in	
Herpes Zoster	2	extremities	2
Hypertension	9	Xanthomatosis	1

The presence of the high incidence of coexisting diseases which are primarily metabolic in nature may be a further indication of the relationship of metabolic dysfunctions and the pertinence of the use of vitamin E in rheumatic diseases.

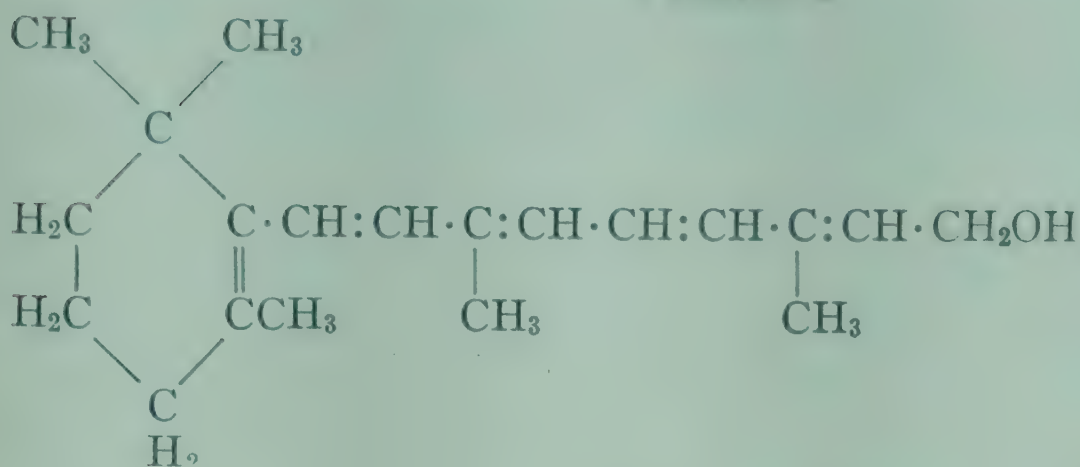
rheumatoid arthritis particularly prodromally and in exacerbation. It appears, from the clinical results achieved, that such metabolic rehabilitation should not be underestimated, for it comprises a therapeutic measure of importance and, moreover, may be striking at one of the etiologic bases of rheumatic entities. The reasonableness of the observation, that the therapeutic benefits which vitamin E confers are due to actual and physiologic improvement in affected muscle and connective tissue, cannot be doubted.

The pharmacological aspect of a drug being the prime interest of one of us (Di Cyan), the known mechanism by which the effect of vitamin E may take place was surveyed. The known mechanism of action of vitamin E (or the tocopherols) does not shed much light on its mode of action in the management of the muscular and connective tissue disabilities attending rheumatic diseases. It has been proposed that vitamin E functions as a coenzyme in the production of acetylcholine.⁴ However, no autonomic effect has been elicited with its use. It apparently has no effect on creatin excretion, which observation would tend to the conclusion that it is not too intimately connected with muscle physiology. It does, however, prevent excessive muscle respiration.⁵ It exerts a protective effect on the liver in poisoning by carbon tetrachloride,⁶ but there are no data on its lipotropic effect in other liver disturbances. It favors the reduction of cholesterol (particularly in conjunction with inositol) but does not seem to affect the course of arteriosclerosis. It is, nevertheless, definitely a biological antioxidant, and its effect as a connective tissue metabolite is due principally to this quality. It is antagonistic to the oxidative effect of calciferol.⁷

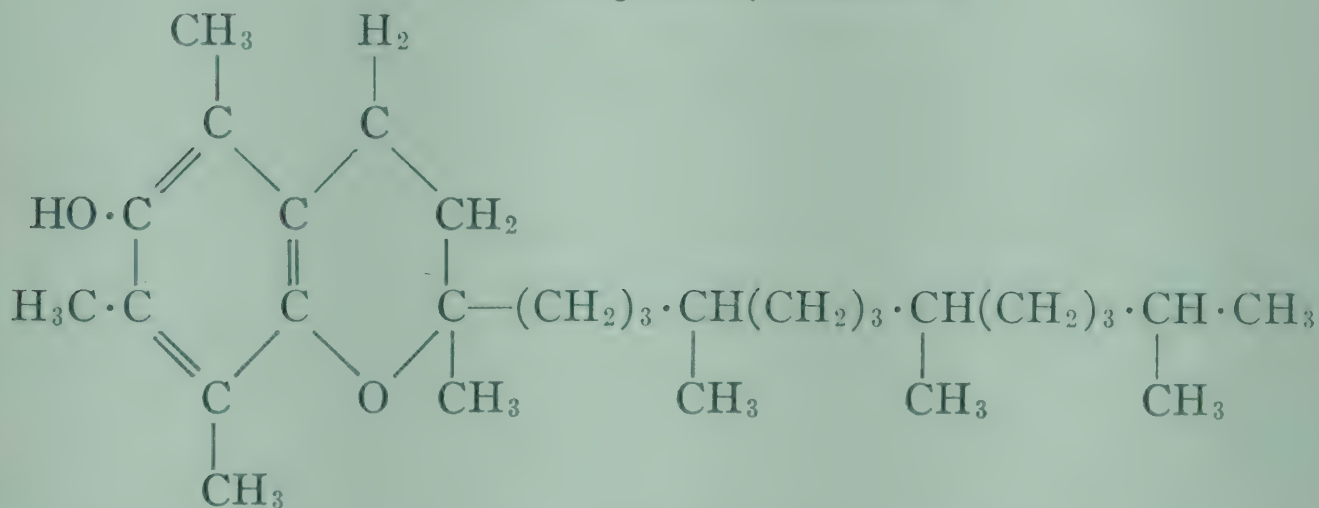
The toxicity of cod-liver oil in vitamin E deficiency⁸ is due to the physiologic incompatibility of vitamins D and E, since vitamin A, the only other vitamin in cod-liver oil, is not antagonistic to it. A consideration of the similarity of the structural formulae of vitamins A and E is interesting in this connection. Both vitamins A and E are derived from unsaponifiable portions, the former industrially from marine animal livers, the latter from vegetable sources. Both have long chain alcohol groups, the former in connection with a β -ionone ring structure and the latter in connection with chroman ring structure. The resemblance, however, ends here.

The studies of Mason & Emmel⁹ on pigmentation anomaly and of Pappenheimer & Victor¹⁰ on deposition of ceroid in vitamin E deficiency would bear out the belief that the effect of vitamin E on the cellular level indicates that vitamin E is a metabolite extremely important for the proper functioning of a number of systems. The direct relationship between the number of functions a vitamin performs and the variety of symptoms of its deficiency is obvious. The change of more than one surrounding variable at a time complicates the proper evaluation of the rôle of such a vitamin.

Vitamin A



α-Tocopherol (Vitamin E)



The propriety of appraising the value of vitamin E therapy in our group of 100 patients was very desirable, since natural periods of remission and psychological amelioration are always experienced by a certain percentage of patients with the use of *any* agent. The use of conjunctive agents in treatment have obscured, in many instances, a definite assay of the role that vitamin E played in clinical improvement. These interfering factors notwithstanding, mitigation or disappearance of pain, increase of mobility of joints, regression of nodules, ridges, and tenderness, and reduction of swelling and recalcification of lesions noted in the successful cases after treatment with vitamin E, both systematically and topically, could hardly be adventitious, as the past history of the patients and past experience in the treatment of these rheumatic entities indicate a different course.

In this series, conjunctive agents used in treatment included one or more of the following: (1) high vitamin E foodstuffs; (2) calcium preparations intravenously; and (3) liver and vitamin B complex intramuscularly. Therapies with which the patients in this series were previously treated consisted

of the measures commonly employed in rheumatic entities, including iodides, salicylates, vitamin D, procaine, physiotherapy, sedatives, analgesics, *etc.*

In a previous series, it was observed that a low blood plasma tocopherol level (BPTL) accompanied clinical findings of certain rheumatic entities. Administration of vitamin E was found to cause a rise of the BPTL and to coincide with clinical improvement. Early cessation of administration of vitamin E in this series was followed by a fall of the BPTL and, shortly thereafter, by a return of the symptoms for which the vitamin E treatment was originally used. Re-administration of vitamin E was followed by the expected increase of the BPTL and then by clinical improvement. Withdrawal of vitamin E after an adequate period of treatment and substitution therefore of a diet high in vitamin E caused a return of symptoms in a comparatively small percentage of individuals, but the lowering of the BPTL was not so marked as that observed when the administration of vitamin E was withdrawn early. Estimating the BPTL¹¹ was found to be suitable from the standpoint of practicability in use, and gave a valuable index of the general level of systemic saturation of vitamin E in the body. TABLE 2 consists of a sample diet, including foodstuffs high in vitamin E.

Clinically, the impression was gained that vitamin D is antagonistic to vitamin E, since longer and more intensive treatment with vitamin E is necessary when vitamin D is administered concurrently. On the same basis, it has been our finding that treatment with calcium is vastly more satisfactory when adequate treatment with vitamin E precedes administration of calcium. We can support this only by clinical observation. We have not been successful in proving this finding by demonstration of increased blood calcium in patients treated with vitamin E. Radiologic evidence indicated degrees of recalcification of decalcified areas when calcium was administered in conjunction with vitamin E. No such evidence was obtained upon the administration of calcium without concurrent or precedent use of vitamin E.

It should be borne in mind that it is too early to assay accurately the rôle of vitamin E in rheumatic diseases, in spite of the fact that definite mitigation or disappearance of pain, increased mobility of joints and, therefore, increased employability of the patient, regression of nodules, ridges, and tenderness, and reduction of temperature and swelling have followed the use of vitamin E in a considerable number of instances. It is indicative, nevertheless, that vitamin E is a valuable adjunct to the treatment of the disturbances of muscle and connective tissue attendant with rheumatic diseases. More is required than a large series of patients for a full evaluation to be made of the rôle of vitamin E in these diseases. It is important that follow-up studies assay the proportion, frequency, duration, and severity of remissions and correlate these findings with the BPTL and the amount of daily intake of vitamin E, both as a drug and in the diet.

Summary

Vitamin E was used topically, orally, and intramuscularly in the treatment of skeletal muscular disorders in rheumatic entities. Results were characterized by amelioration of pain, mitigation or disappearance of physical

TABLE 2
SAMPLE OF A COMPLETE DIET, INCLUDING FOODS HIGH IN VITAMIN E

	Amt. gm.	CHO gm.	Prot. gm.	Fat. gm.	FE mg.
<i>Breakfast:</i>					
1 slice orange	120	12.0			.30
$\frac{1}{2}$ cup cereal, cooked with	100	12.0	2.0	.5	.60
*1 tbsp. wheat germ	15	7.4	3.7	1.5	
1 oz. cream	30	1.0	1.0	12.0	.07
1 slice toast	30	15.0	3.0	1.0	.03
1 tbsp. butter	15			12.5	.036
1 glass milk	200	10.0	7.0	8.0	.16
<i>Lunch:</i> salad consisting of					
2 hard boiled eggs			12.0	12.0	2.30
* $\frac{1}{4}$ head lettuce	100	3.0	1.0		.58
1 slice tomato	100	3.0	1.0		.43
$\frac{1}{2}$ cup green soy beans	100	6.0	12.5	6.5	
*1 oz. dressing made with					
1 tsp. peanut oil	15			15.0	
1 sl. bread	30	15.0	3.0	1.0	.03
1 tsp. butter	5			4.1	.012
*1 banana, sliced, with	200	42.0			.82
1 oz. cream	30	1.0	1.0	12.0	.07
<i>Dinner:</i>					
$\frac{1}{2}$ cup sectioned orange					
and grapefruit	100	10.0			.25
*4 oz. lean beef	120		32.0	20.0	1.7
1 medium potato	100	19.7	2.0		.48
* $\frac{1}{2}$ cup spinach	100	3.0	1.0		4.0
*1 head lettuce with	100	3.0	1.0		.58
dressing (1 tsp. peanut oil)	15			15.0	
$\frac{1}{2}$ cup chocolate pudding	100	21.2	3.3	3.8	0.35
1 cup tea with lemon					
2 tsps. sugar	10	10.0			
<i>Bed-Time:</i>					
1 glass milk	200	10.0	7.0	8.0	.16
Total for Day:	1935	204.3	93.5	132.9	13.836

Calories: 2387
* Comparatively high in v tamin E.

stigmata, and increased mobility of the joints. The rôle of vitamin E in the treatment of these entities can be properly evaluated only after additional studies, comprising additional cases, and adequate follow-up are completed. Present evidence warrants the consideration of vitamin E as a connective tissue vitamin, and projected pharmacological work could properly be in that direction. Perhaps techniques employing isotopic elements may be productive in evaluating the full rôle of vitamin E.

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VITAMIN E AND COLLAGEN IN THE RHEUMATIC DISEASES

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The rheumatic diseases are associated with abnormal deviation of the connective tissue. These diseases are rheumatic fever, disseminated lupus erythematosus, dermatomyositis, periarteritis nodosa, diffuse scleroderma, and, possibly, rheumatoid arthritis and fibrositis. There seems to be increasing evidence linking the connective tissue in general and interfibrillar material in particular with rheumatic diseases.

The connective tissue consists of two distinct components: the fibrillar material and the interfibrillar substance. Macromolecular (electron microscopic) studies have shown that the fibrillar substance has specific cross striations in fish, amphibia, and mammals. These cross striations occur at a regular axial periodicity of 640 angstrom units. The fibrils branch like the branches of a tree. At least two components are present in the interfibrillar substance: the amorphous and viscous ground substance, and the cement substance proper. The connective tissue or fibrous elements are embedded in the cement substance. The fibrous elements are made up of collagen, reticulin, and elastic fibers. These fibers are fibrous proteins of very high molecular weight. X-ray diffraction and electron microscopic studies of collagen fibers have shown that they are composed of branched fibrils which have alternating bands of higher and lower density spaced at regular distances from each other. The heating of collagen fibers in aqueous solution results in amorphous gelatin. It is interesting that collagen fibers of all mammals have the same structure. Embryonic collagen fibers are soluble in salt-free dilute acid and, when these solutions are treated with salt or neutralized, the proteins precipitate as fibers which possess the same fine structure as the native fibers.

The cement substance contains both protein and mucopolysaccharides. Very little is known about the protein. More is known about the mucopolysaccharides. Of the four known mucopolysaccharides present in the cement substance, hyaluronic acid has received the most attention. The exact structure of the mucopolysaccharides is unknown. However, the molecular weight of hyaluronic acid is quite high. The substance occurs in group A and group C hemolytic streptococci, in embryonic tissue, synovial fluid, skin, and in the humors of the eye. This acid is hydrolyzed by a specific enzyme known as hyaluronidase. The chief experimental sources for hyaluronidase have been various hemolytic streptococci and the testes of bulls. Hyaluronidase has been known as the "spreading" factor. In recent clinical practice, it has been shown that the addition of 0.1 microgram of this substance to fluids to be given intramuscularly greatly increases the "spreading" of this fluid and causes the rapid absorption of large quantities of fluid with a minimal amount of discomfort. Suggestions have been made that this substance, in some way liberated by hemolytic streptococci, inter-

feres with the cement substance of connective tissue by breaking down the hyaluronic acid and initiating the pathological picture of the rheumatic state. This probably is one mode of initiation of deviation from the normal histology of connective tissue.

Steinberg has shown previously that the pathology of primary fibrositis and nutritional muscle dystrophy were strikingly similar. This muscular dystrophy was first described in 1931 by Goettsch and Pappenheimer in rabbits and guinea pigs. The early stages of nutritional muscular dystrophy show marked interstitial edema. Marked inflammatory reaction with polymorphonuclears is present. The edema soon disappears and the polymorphonuclears are replaced by mononuclear histiocytes. This is followed by calcification of the necrotic fibers. Degeneration of cross-striated musculature in rats whose diets were low in vitamin E was described by Evans *et al.* Electron microscopic studies would be of value in determining the changes present in the cement substance in these conditions. Since these conditions are initiated in Vitamin E-deficient animals, it is logical to assume that vitamin E is necessary for the normal nutrition of this substance.

There has been some clinical experience with the use of vitamin E in the treatment of disseminated lupus erythematosus, diffuse scleroderma, dermatomyositis, rheumatic fever, and primary fibrositis. No clinical improvement has been noted in four cases of disseminated lupus erythematosus, three cases of diffuse scleroderma, and two cases of dermatomyositis. Definite clinical improvement has occurred in the treatment of patients with primary fibrositis (particularly Dupuytren's contracture and Peyronie's disease), and questionable clinical improvement has occurred in patients with rheumatic fever.

A disorder of the connective tissue on a metabolic basis may result from: (a) an insufficient intake of vitamin E; (b) a sufficient intake but an abnormal absorption of vitamin E; and (c) a normal intake and a normal absorption but an abnormal use by the connective tissue of vitamin E. Primary fibrositis may result from any one of these three conditions. It has been shown previously that the blood vitamin E level in primary fibrositis is usually normal (normal value range between 0.9 and 1.6 mg. per 100 cc.).

On rare occasions an individual's diet is inadequate in vitamin E and changes in the connective tissue structure occur. One such patient was reported in 1947. A 56 year old white man had a sufficiently poor intake of vitamin E to lower his initial vitamin E blood level to 0.79 mg. per cent. A biopsy of the biceps muscle revealed occasional degeneration of muscle fibers with abnormal arrangement of the nuclei. An occasional muscle giant cell was noticed. Rare areas of lymphocytic infiltrations were present. The blood vitamin E level rose to 0.91 mg. per cent after two weeks daily of 300 mg. of mixed natural tocopherols. A repeat biopsy four weeks after the initial one revealed improvement. The muscle fibers were swollen and homogeneous and the striations, while present, were faint. There was slight peripherization of nuclei and no round cell infiltration was noted. There was marked clinical improvement in this patient.

The second method of metabolic disturbance, is seen in cases of cirrhosis

of the liver. The intake of vitamin E is normal and ample but the absorption is poor.

Dupuytren's contracture is a form of fibrositis and represents the third method of metabolic disorder. The condition varies from mild fibrosis of the palmar fascia involving one digit to that involving all digits, with extreme contracture resulting in a flexion deformity of the hand. The thumb is the only flexor tendon that usually remains uninvolved. The flexor tendons of the third and fourth fingers are most frequently involved. The skin becomes dimpled as a result of the contracture and very often becomes leathery or sclerodermatous in nature. On rare occasions, both the palmar fascia and the plantar fascia are involved.

Scott and Scardino recently called attention to the concurrence of Dupuytren's contracture and Peyronie's disease. The latter condition is characterized by fibrous infiltration of the intercavernous septum of the penis. This fibrosis usually results in the formation of plaques. This condition results in the curvature of the penis on erection and makes intromission difficult or impossible. These authors reported six cases of Dupuytren's contracture associated with twenty-three cases of Peyronie's disease. This is further evidence of a general metabolic disturbance of connective tissue in primary fibrositis. Forty cases of Dupuytren's contracture have been adequately followed by the writer in recent years so that an evaluation of treatment appears justified. Two cases of Peyronie's disease have been observed by the writer under similar circumstances.

Failures

Three of the 40 patients with Dupuytren's contracture treated with vitamin E were total failures. One such patient has been followed with large doses of vitamin E (300 to 400 mg. of mixed natural tocopherols daily for one year). He also had a period of two months treatment with alpha-tocopherol di-sodium phosphate in a dose of 300 mg. daily without clinical benefit. His initial vitamin E levels were normal. Another failure was a young physician, aged 29, who had involvement of both his palmar and his plantar fascia. Signs of the disease had been present for ten years. Follow-up of two months was inadequate. A third failure concerned a physician who took mixed natural tocopherols over a period of twelve months without clinical benefit. He had a very advanced bilateral contracture extending over a period of many years. The remaining 37 patients manifested marked to complete clinical benefit.

Experience has taught that failure of treatment with vitamin E at the end of two to three months need not be conclusive. A case in point is that of G. R., a white male aged 57, first seen 4/21/47 with a bilateral Dupuytren's contracture. He was given 300 mg. of mixed natural tocopherols daily from 4/21/47 until 3/27/48 without improvement. He was then placed on three capsules daily of concentrated mixed natural tocopherols each capsule containing 150 mg. of mixed natural tocopherols which in turn contained 75 mg. of dl, alpha-tocopherol. Marked clinical improvement was first noted 2/12/49.

No doubt some initial failures will respond to intensive prolonged therapy. Other cases of Dupuytren's contracture may require surgery. However, the high recurrent rate after surgery indicates that tocopherol therapy should be added to surgical treatment. In all surgically treated cases, mixed natural tocopherols should be given postoperatively to avoid recurrence.

Peyronie's Disease

Of the 2 patients with Peyronie's disease, one had complete disappearance of the plaque after taking 100 capsules, each containing 50 mg. of mixed natural tocopherols. The condition had been present for a period of two months.

Another male, D. D., has had a marked curvature of the penis on erection during the past three months. He has been on mixed natural tocopherols for a period of three months and no clinical benefit has occurred.

Primary Fibrositis (Generalized Involvement)

Generalized primary fibrositis is not rare and is more common in the fifth and sixth decades of life. The disease is equally common in both sexes. A patient so afflicted presents the objective picture of good health after years of involvement.

The complaint is one of muscle soreness, usually occurring in one or several groups of muscles. Chilling or unusual use of muscles results in extreme soreness and lameness. A twenty-four hour creatine urinary excretion is elevated beyond 100 mg. in a twenty-four hour period in systemic involvement. If the symptoms are localized to the palmar fascia, however, as in Dupuytren's contracture, the creatine excretion is usually below 100 mg. Sedation with one of the barbiturates results in no improvement in the muscle soreness. This sedative test distinguishes the disease from psychosomatic rheumatism. The sedimentation rate, white blood count, and anti-streptolysin titers are normal in this condition. Mixed natural tocopherols in a dose of 300 mg. daily, given in three equally divided doses after meals, has proved effective in a vast majority of such cases. A maintenance dose must be continued after clinical cure has been obtained. This dose varies from 50 to 150 mg. in most cases and, on rare instances, 300 mg. must be continued daily indefinitely.

Untoward symptoms from the use of mixed natural tocopherols extending over a period of years have not been manifested. On rare occasions, mild gastric irritation results from the oil solution. Neither pure alpha-tocopherol in oil nor mixed natural tocopherol should be given intramuscularly in these cases. The mixed natural tocopherol produces severe local reactions. Approximately three to six months after giving pure alpha-tocopherol in oil, oleogranulomas developed in approximately 50 per cent of cases. In no instance in which pure alpha-tocopherol has been given to progressive cases of muscular dystrophy have oleogranulomas developed. This indicates the abnormal reaction of the fibrous connective tissue in patients suffering from primary fibrositis.

Three hundred cases of this condition have been treated by the writer in

the past ten years. A muscle or group of muscles are usually involved. Often, the patient gives a history of recurrent wry neck or presents a picture of temporary curvature of the spine upon exposure to drafts or moderate stress. Radiographs of the spine are essentially negative and the sedimentation rate is normal. Various bursae may become bothersome on slight trauma. Thus, a physician, aged 48, develops bursitis of the gluteal group after a short hunting trip, or subacromial bursitis after nine holes of golf. A school teacher, aged 52, develops sore muscles of the lower extremities after standing while teaching for four hours. A business executive, aged 58, is seen with a marked list of the spinal column after sleeping in a cold pullman car.

Rheumatic Fever

The exact cause of rheumatic fever is unknown. The classical history of rheumatic fever runs the following course. Sore throat appears. Hemolytic streptococci of the beta type are cultured from the throat of the infected individual. The sore throat disappears after a number of days and is followed by a silent period of three to four weeks. The silent period is broken by a rheumatic state characterized by migratory, hot, swollen joints. The average duration of this period is six weeks. The well-established fact in the control and management of this condition has been established. Sulfadiazine in a dose of 0.5 gm. taken daily will, in the vast majority of cases, prevent reinfection with hemolytic streptococci and, therefore, reactivation of the rheumatism. Once the rheumatism has started, sulfadiazine is of no value and may be harmful. The rheumatic stage is symptomatically improved by the use of salicylates. Salicylates have been shown to inhibit hyaluronidase, the "spreading" factor.

The characteristic lesions of rheumatic fever (Aschoff bodies) are found chiefly in the perivascular connective tissue space of the myocardium, in the connective tissue layer of the endocardial and serous surfaces, and much less commonly in the walls of arteries. The first pathological change noted in this connective tissue is fibrinoid degeneration. This pathological change in connective tissue is evidenced by swelling and eosinophilia and refractivity of the fibers. Similar changes in the ground substance are sometimes present. The occurrence and type of fibroblastic reaction in the areas in which the collagen is altered vary in the several rheumatic diseases. The cellular reaction appears more characteristic than the changes in the noncellular elements in rheumatic fever. These changes in rheumatic fever are sometimes limited exclusively to the walls of blood vessels, whereas, in disseminated lupus erythematosus and diffuse scleroderma, they may be found in many tissues and organs of the body. The primary change in the infected locations in rheumatic fever is necrosis or fibrinoid degeneration of small areas of collagen accompanied simultaneously by a striking characteristic cellular response. Fibroblastic proliferation is progressive. Giant cells appear and, almost from the beginning, the inflammatory process is associated with the production of new fibrous tissue, especially in the cardiac valves.

The involvement of collagen tissues and perhaps the ground substance in

rheumatic fever stimulated the speaker to assay the value of vitamin E in this condition. The case histories which follow are presented with these suppositions in mind.

Case I. (F. B.) A white male, aged 12, was first seen March 1, 1947, at which time a diagnosis of rheumatic fever and rheumatic carditis was made. He had had his first attack of rheumatic fever two years before. He had painful swollen migratory joints for two months which were preceded for several weeks by the typical rheumatic sore throat. No symptoms were experienced until December, 1946, at which time he began to run a low grade temperature of 100 to 101 degrees. Joint symptoms reappeared in the middle of February, 1947. At that time, he began to have migratory painful swollen joints. Again, the episode of rheumatism was preceded by a sore throat several weeks before its onset. When seen March 1, 1947, he was running a low grade fever of 99 to 102. A grade four systolic murmur was heard along the left sternal border. It was most pronounced in the third interspace. No cardiac enlargement was noticed under the fluoroscope. An electrocardiogram revealed a P-R interval of 0.18 seconds. All leads were normal, except for the following slight deviations from normal: there was a deep S₁ (50 per cent negative deflection); an inverted T₃ wave was present; and the sedimentation rate was 25 mm. per hour. He was placed on 300 mg. of mixed natural tocopherol daily March 1, 1947. His sedimentation rate was 17 mm. on March 10, 23 mm. on March 23, 21 mm. on March 31, and remained normal on April 7, April 14, April 26, May 5, May 10, and May 31, (all 1947). It rose slightly to 16 mm. on June 21, 1947, and to 17 mm. on July 26, 1947, and then returned to normal the next week. Unfortunately, vitamin studies on his blood were not done until seven days after the vitamin E therapy had been started. The following values were then obtained; carotene 266 gamma per cent; vitamin A 230 micrograms; and vitamin E 1.48 mg. per cent. On April 14, 1947, a questionable early presystolic murmur was heard at the apex. On April 26, 1947, there was no question of the presence of the presystolic murmur at the apex. Other blood studies that might be of interest were two antistreptolysin titres done March 23, 1948, and June 8, 1948 (note that this was one year after the patient was first seen). Both values were still 250 ASL units.

Several interesting clinical incidents were that the patient had four severe nose bleeds about ten days after starting vitamin E therapy. On August 30, 1947, he had a very severe sore throat, and at this time penicillin, in a dose of 50,000 units three times daily by mouth, was given for three days. No exacerbation of the rheumatism occurred after this sore throat. He had been on 150 mg. of mixed natural tocopherols daily since the second month of institution of therapy. When last seen March 23, 1948, he had a classical presystolic murmur at the apex and a short diastolic at the aorta area.

The clinical course followed in this case could well be that of a natural history of rheumatic fever. Certainly, the vitamin E did not prevent the scar formation in both the aortic and mitral valve which later evidently occurred, judging by the cardiac findings. The most striking result with

vitamin E therapy in this case was the stopping of joint symptoms after about one week of therapy.

Case II. (M. B.) This case shows that the blood vitamin E in rheumatic fever may be normal. M. B., a female aged 20, was first seen February 2 1947. She had had her first attack of rheumatic fever at the age of 3. She had had recurrent muscle and joint aches ever since. The physical examination revealed a classical presystolic murmur at the apex and an aortic diastolic murmur which was heard loudest at the 3rd left intercostal space. The electrocardiogram was within normal range and the P-R interval was 0.18 seconds. Fluoroscopic examination revealed no cardiac enlargement and her sedimentation rate was 13 mm. per hour. Blood studies before the institution of vitamin E showed a blood level of 1.01 mg. per cent. Blood studies one week after vitamin E therapy were carotene 161 gamma per cent, vitamin A 202 micrograms per cent, and vitamin E 1.30 mg. per cent. When last seen, March 10, 1947, approximately two weeks after institution of therapy, the patient's joint symptoms had disappeared. A note from her at the end of 1947 revealed continued relief of joint symptoms. Her sedimentation rate when last seen was practically unchanged at 16 mm. per hour. Thus, symptomatic relief occurred with vitamin E therapy.

Case III. (E. F.) A white male, aged 8, had been in a rheumatic state for three years. The rheumatic state was initiated at the onset by a history of a severe cold and sore throat. He had run a low-grade fever and had ankle aches and nose bleeds ever since. Also, he has had rheumatic nodules in the fingers and toes. He has a sister, aged 15, who has had rheumatic fever off and on for ten years. The chief physical findings were a low-grade fever of 99.4° F., a rapid pulse rate of 102, and a systolic murmur heard over the entire precordium but loudest in the pulmonic area. The electrocardiogram was normal in all four leads and the P-R interval was 0.18 seconds. Fluoroscopic examination revealed no cardiac enlargement.

The initial blood studies taken May 20, 1947, before vitamin E therapy, were carotene 240 gamma per cent and vitamin E 0.78 mg. per cent. He was placed on 300 mg. of fixed natural tocopherols daily and the follow-up on his vitamin blood studies was as follows: May 27, 1947, one week after institution of therapy, the vitamin E blood level was 1.28 mg. per cent, June 3, 1947 it was 1.03 per cent, with vitamin A, 142 units and carotene, 178 gamma per cent. On June 17, 1947 the blood vitamin E level had risen to 1.50 mg. per cent. His leg aches improved considerably by June 3, 1947, and none were present on June 18, 1947. The heart murmurs diminished in intensity so that they were difficult to hear at this date. He was next seen on July 1, 1947, and in a week's time he began to run a low-grade fever and the muscle and joint symptoms became more pronounced. Hemolytic streptococci were grown from the throat on July 8, 1947, and again on July 15, 1947. There was constant diminution in the number of hemolytic streptococci found in the throat. He was continued on 300 mg. of mixed natural tocopherol and when last seen, July 15, 1947, the murmur became

more pronounced both at the apex and aortic areas. The mother lost interest in our scientific investigation and the patient was not seen again.

This case seems to indicate the ineffectiveness of vitamin E in controlling the rheumatic mechanism produced by hemolytic streptococci in rheumatic fever. In retrospect, the wise thing would have been to institute antibiotic therapy at the point at which hemolytic streptococci appeared in the throat. The sedimentation rates from this boy on May 27, June 3, June 18, and July 1, 1947, were all normal. The highest rate, 13 mm. per hour, was on June 3, 1947. Another interesting finding was that of a low blood vitamin E level before institution of therapy.

Case IV. (J. S.) A white female, aged 12, was first seen October 18, 1944. She had a five-year history of rheumatic fever. She had had four attacks of rheumatic fever during this period. When first seen, she had a five-months' history of painful swollen joints. Her sedimentation rate was elevated at 26 mm. per hour and she had a grade II apical systolic murmur. No electrocardiogram was done. She was placed on salicylate therapy. She has remained on salicylate therapy during the cold months of the year ever since. On rest and salicylate therapy, her sedimentation rate dropped to normal on November 1, 1944, and remained so until October 11, 1947. A systolic murmur was also associated with a presystolic murmur, which was first heard on October 25, 1944. Both of these murmurs gradually disappeared and, at the present time, they are not heard (she was last seen September 17, 1948). The sedimentation rate, which rose in October, 1947, remained elevated until January 24, 1948, at which time it dropped again. It has remained normal ever since. On January 17, 1948, she was first placed on tocopherol therapy. On that date the sedimentation rate was 25 mm. per hour. When seen one week later, January 24, the sedimentation rate had dropped to 13 mm. per hour and has stayed down since. In other words, the sedimentation rate, which rose October 11, 1947, while on salicylate therapy, remained elevated January 3, 1948, and January 17, 1948. With additive tocopherol therapy it dropped to normal on January 24, 1948. This may be interpreted as indicating that tocopherol therapy aided in the dropping of the sedimentation rate or else it could be a natural course of events. No blood vitamin E studies were done on this patient.

The foregoing studies, which are cases selected from a series of 15 patients, suggest the possibility that vitamin E is of some value in the treatment of rheumatic fever. These studies also indicate that vitamin E is ineffective in preventing attacks of rheumatic fever when hemolytic streptococci are present in the throat. The antibiotics useful in these cases should be used whenever the individual has a so-called rheumatic sore throat.

Case V. (C. A. DeC.) A white female, aged 6, was first seen January 22, 1949. She gave a history of having run a low grade fever of 99 to 102° F from June to December, 1947. Her physician informed the parents that the child had rheumatic fever and that a heart murmur was present. In December, 1947, she was placed on prophylactic sulfadiazine. A few weeks

later, she began to run a low grade fever 99 to 99.2° F and to feel listless. Constant fatigue was present. No joint symptoms and no skin rash were present. The patient had had no recent upper respiratory infections. She had stopped her sulfadiazine (0.5 gr.) about one week after beginning to run fever in December of 1948.

When first seen, January 22, 1949, the patient appeared listless and complained of fatigue and generalized aches and pains. Her temperature was 98.6 and her pulse was 78. A grade II aortic systolic murmur was present. Her electrocardiogram was normal and the PR interval was 0.18 seconds. Fluoroscopic examination of the heart and lungs was negative. An anti-streptolysin titer done January 27, 1949, was 50 ASL units. She was placed on 150 mg. mixed natural tocopherols daily. Also, two abscessed teeth were removed. She became afebrile on February 12, 1949, one week after removal of the last infected tooth. Her clinical improvement has been maintained to the date of writing, March 5, 1949. The murmurs have disappeared. A repeat antistreptolysin February 23, 1949, was 50 ASL units. The white blood count on January 22, 1949, was 16,250 and the sedimentation rate 15 mm. per hour (corrected to a 46 hematocrit). The white blood count dropped to 10,400 on February 12, 1949, and the sedimentation rate to 9 mm. per hour. The throat culture January 22, 1949, revealed no hemolytic streptococci.

This case indicates the difficulty in evaluation of therapy in rheumatic fever. It is perhaps possible that the clinical improvement and the previous untoward symptoms were due to the infected tooth and its treatment, rather than to rheumatic fever and its therapy. However, the disappearance of the heart murmur is interesting and challenging.

Summary and Conclusions

(1) Abnormal metabolism of fibrous connective tissue may result from Vitamin E deprivation. This deprivation may occur as result of a lack of Vitamin E intake (rarely), a normal intake and a normal absorption of Vitamin E but lack of utilization of tissues of this vitamin (common), and by normal intake but poor absorption (cirrhosis of the liver), resulting in a low blood level of all the fat soluble vitamins including Vitamin E.

(2) Primary fibrositis may manifest itself as Dupuytren's contracture, Peyronie's disease, and as a generalized systemic involvement of the muscles.

(3) Creatinuria is common in the generalized systemic involvement of primary fibrositis.

(4) The blood level of Vitamin E is usually normal in primary fibrositis but the utilization curve is the plateau pattern, indicating difficulty in utilization.

(5) The traumatic theory of the cause of Dupuytren's contracture is untenable in view of the afore-mentioned findings.

(6) Mixed natural tocopherols in a dose of 300 mg. daily, divided into three equal doses after meals, is clinically effective in the treatment of primary fibrositis.

(7) A maintenance dose must be continued to prevent recurrence of the syndrome.

(8) Mixed natural tocopherols taken orally have practically no toxicity in human individuals in the doses recommended.

(9) Neither mixed natural tocopherol nor pure alpha-tocopherol should be given parenterally to patients suffering from primary fibrositis. Dangers of such medication have been described.

(10) Vitamin E may be of value in the treatment of rheumatic fever.

(11) Vitamin E is of no value in treatment of the para-rheumatic diseases.

(12) Histo-chemical studies of vitamin E in the rheumatic diseases may aid in the discovery of more effective therapy.

THE USE OF TOCOPHEROLS IN THE TREATMENT OF PEYRONIE'S DISEASE

By Peter L. Scardino and William Wallace Scott

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Since reporting a new concept in the treatment of Peyronie's disease in November 1947,¹ the authors have continued to treat chronic cavernositis with tocopherols with encouraging effectiveness. The original group of 23 cases has been reviewed and is reported as a follow-up study. In addition, 10 new cases, similarly treated and not previously reported, are presented.

Peyronie's disease is, pathologically, a fibrous replacement of the intercavernous septum of the penis. The fibrosis may extend into Buck's fascia and the tunica albuginea on either side of the septum and result in the formation of plaques. Involvement of the corpus spongiosum does not occur. Formation of the plaques frequently results in penile curvature and painful erections, making sexual intercourse difficult or impossible.

Previous reports^{1, 2, 3, 4} of the efficacy of the tocopherols in the treatment of various forms of fibrositis suggested to Scott the use of tocopherols in Peyronie's disease, also a form of primary fibrositis. In January, 1947, the authors began the administration of the tocopherols to patients with Peyronie's disease. During the ensuing 10 months, 23 patients were treated with 500 milligrams of mixed tocopherols or 200 mg. of synthetic alpha-tocopherols, without toxic manifestations.

The incidence of symptoms and signs before treatment in the first group of 23 cases of Peyronie's disease is presented in TABLE 1. From inspection of this table it is apparent that the most common symptom was penile curvature. Painful erections occurred in twelve cases, with loss of libido in four cases and loss of potentia in sixteen. Dupuytren's contracture was present in six cases, and penile plaques were found in all but one patient. Most of the patients noted the onset of the disease during the fifth decade.

The results of the therapy employed are presented in TABLE 2. Inspection of this table reveals that a complete disappearance of curvature occurred in four cases, with some change in all but five of the patients. Pain disappeared in all cases, and sexual intercourse became normal for ten. Change was noted in the palmar contracture of four of the cases, with marked improvement in one. Objective evidence of response to therapy was measured by the change in penile plaques. In this group, all but two patients showed changes in the size, shape, and consistency of the penile plaques.

A follow-up study of these twenty-three patients was undertaken to determine their status at the time of the present report. Two particular aspects of the problem were investigated: (a) treatment required to obtain response; and (b) recurrence of signs and symptoms after cessation of therapy. Subjective response, i.e., relief of pain, was the first symptom notably affected by the drug. Three hundred mg. of mixed tocopherols elicited a change in over 60 per cent of the patients within 30-60 days after therapy

TABLE 1
THE INCIDENCE OF SYMPTOMS AND SIGNS BEFORE TREATMENT IN TWENTY-THREE CASES
OF PEYRONIE'S DISEASE
GROUP I

Symptoms	Incidence	Signs	Incidence
Penile curvature		Penile plaques	
Severe	11	Severe	6
Moderate	9	Moderate	16
Not present	3	(Collar)	1
Pain on erection	12	Dupuytren's contracture	6
Loss of libido	4		
Loss of potentia	16		
Palmar contractures	6		

TABLE 2
THE INCIDENCE OF CHANGE IN SYMPTOMS AND SIGNS AT TWENTY-THREE CASES OF
PEYRONIE'S DISEASE TREATED WITH TOCOPHEROL
GROUP I

Symptoms	Incidence	Signs	Incidence
Penile curvature		Penile plaques	
Disappearance	4	Marked decrease	6
Marked decrease	4	Moderate decrease	15
Moderate decrease	10	No change	2
No change	2		
No mention	3	Dupuytren's contracture	
Pain on erection		Marked improvement	1
Disappearance	12	Moderate improvement	3
		No change	2
Sexual intercourse			
Return to normal	10		
Unsatisfactory	3		

was initiated. Only infrequently, by increasing the dosage or changing the type of tocopherol, was better progress made in the treatment of the disease. While a majority of the patients had noted the presence of the disease for less than a year, the occasional patient had had the disease for a longer period. In spite of previous forms of therapy applied to the diseased organ, it was noted that the longer the disease had existed, the more resistant it was to tocopherol therapy. None of the patients received the drug longer than 18 months. Recurrence of signs or symptoms has not been reported after cessation of therapy. Whatever progress is made is apparently maintained without continuous therapy.

Of the 23 patients in Group I, a one-year follow-up study has been complete on 17. Of the 17, nine could still be classified as good results, five fair, and three unchanged (TABLE 3). A follow-up study on the other six

cases in this group was unsatisfactory. One patient in the initial study, where response was rated fair, was found on further evaluation in the follow-up study to have shown no response to therapy. This accounts for the apparent discrepancy in the overall results in Group I (TABLE 3). This pa-

TABLE 3
GROUP I

<i>Results: May, 1942</i>		<i>Results: April, 1949</i>	
Total No. Cases	13	Total No. Cases Followed	17
Results:		Results:	
Good	11	Good	9
Fair	10	Fair	5
Unchanged	2	Unchanged	3

tient's initial response was purely symptomatic and, on closer questioning, failed to satisfy our criteria for response. In the Group I follow-up, 14 patients, of the 17 followed, continued to demonstrate response to therapy, for an overall response of 82 per cent. However, only 55 per cent were good responses.

Encouraged by the first group of patients, we continued to administer tocopherols to all cases of Peyronie's disease which came to our attention. Group II consists of ten patients whose courses have been tabulated, and an evaluation has been made by comparing pre-treatment symptoms and signs with those observed after treatment (TABLES 4 and 5). It will be

TABLE 4
THE INCIDENCE OF SYMPTOMS AND SIGNS BEFORE TREATMENT IN TEN CASES OF
PEYRONIE'S DISEASE
GROUP II

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature		Penile plaques	
Severe	5	Severe	7
Moderate	4	Moderate	3
Not present	1	Dupuytren's contracture	1
Pain on erection	5		
Loss of libido	2		
Loss of potentia	6		
Palmar contracture	1		

noted that penile curvature was present in all but one patient. Painful erections were reported by five patients with loss of libido by two and of potentia by six. Palpable penile plaques were present in all cases, varying from moderate in three to severe in seven patients. With treatment, which was similar to that of the first group, response was shown by a decrease in penile curvature in all but two patients. Pain disappeared in all who pre-

TABLE 5

THE INCIDENCE OF CHANGE IN SYMPTOMS AND SIGNS IN TEN CASES OF PEYRONIE'S DISEASE TREATED WITH TOCOPHEROLS

GROUP II

<i>Symptoms</i>	<i>Incidence</i>	<i>Signs</i>	<i>Incidence</i>
Penile curvature			
Disappearance	2	Penile plaques	
Marked decrease	3	Marked decrease	3
Moderate decrease	3	Moderate decrease	3
No mention	2	No change	4
Pain on erection		Dupuytren's contracture	
Disappearance	5	No change	1
Sexual intercourse			
Return to normal	5		
Unsatisfactory	2		
No mention	3		

viously reported this symptom. Five patients reported a return to normal sexual intercourse. Definite change was noted in 70 per cent of the penile plaques, with marked decrease in size and consistency in one.

In an overall evaluation (TABLE 6), we have rated the response as good in

TABLE 6
Response to Therapy

	<i>Group II</i>	<i>Groups I and II</i> (Only cases followed included)
Good	4	13
Fair	4	9
No change	2	5

four cases, fair in four cases, and no change in two. Of the two groups in TABLE 6, we were able to report 13 good cases, 9 fair, and 5 without response to therapy.

The authors did not utilize concurrent placebo studies. The nature of the problem was such that an evaluation could be accurately determined by objective evidence, obviating the necessity for a placebo study. The subjective evidence is, as is all subjective evidence, open to criticism. It will be noted (TABLE 2) that in Group I the initial subjective response, *i.e.*, penile curvature as reported by the patient, occurred in 18 of the 23 patients. Pain on erection disappeared in 12 out of 12 patients who complained of this symptom. However, the objective finding of penile plaques in 23 patients, of which 21 showed demonstrable changes in the size, shape, and consistency by palpation and objective caliper measurement, was sufficient supporting evidence to warrant our conclusions (TABLE 3). Likewise, in Group II, comparable subjective and objective results were obtained.

The pathogenesis of chronic cavernositis is poorly understood. The

physiology of the tocopherols in effecting a response in Peyronie's disease is, apparently, similar to the action of the tocopherols in other forms of primary fibrositis.

Summary and Conclusions

Twenty-three cases of Peyronie's disease previously reported are reviewed, and a two year follow-up study is presented. Ten additional cases, not previously reported, are reviewed.

Of the 17 cases followed and the 10 new cases reported, 13 have been classified as good results, 9 fair, and 5 showed no response to therapy.

Eighty-one per cent showed at least some response to tocopherol therapy. Somewhat over 48 per cent of the patients showed a good response. These findings warrant the further use of tocopherols in the treatment of Peyronie's disease.

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Discussion of the Papers

DR. MORRIS ANT, M.D. (*Brooklyn, N. Y.*): In discussing the preceding three papers, I will begin with our own presentation, read by Dr. Di Cyan. In our 100 cases, there were no acute rheumatic fevers. We had cases of rheumatoid arthritis, osteoarthritis, fibrositis and fibromyositis or muscular rheumatism. All cases had been under treatment by other physicians with various therapies and, in fact, 90 per cent of the patients were sent to me by them with the hope that institution of a metabolic regimen would help in their management.

My first instructions were to stop all physiotherapy on the premise that while physiotherapy increases the blood supply to a joint or muscle area, it also increases temperature, congestion, stasis, and even edema. Patients were advised to follow a high vitamin E diet. A sample diet is shown in our paper. This diet should be adjusted to suit individual requirements.

Depending on the degree of arthritic involvement such as pain, swelling of muscle groups, fibromyositic nodules or induration, topical vitamin E was applied in the form of an ointment. Then, natural mixed tocopherols 200 mg. to 1,000 mg. daily were prescribed. Eventually, doses were reduced down to 200 mg. daily. No gastric symptoms developed. Finally, injectable vitamin E was used, wherever I thought it might aid in storing larger quantities of the vitamin. No indurations or abscesses were noted.

A few patients who were treated with vitamin D were permitted to continue with it, only to find that by taking vitamins D and E simultaneously, they developed gastric irritation. When vitamin D was stopped and vita-

min E continued, gastric symptoms were gone. Wherever x-rays showed calcium absorption, calcium was added intravenously to the regimen with signs of recalcification, whereas when calcium was given previously without vitamin E, recalcification was not apparent.

In osteoarthritis, criteria for clinical improvement were reduction of swelling, and improvement of motility, and in cases of rheumatoid arthritis a reduction of spindle shaped arthritic changes with return to normal color and appearance. Early in the use of vitamin E, I observed its dehydrating effect. In applying the ointment or wheat germ oil to swollen muscle areas, small films or droplets of water would form, with reduction in the swelling, while with external application of rubifacients no such phenomenon was noted. In cases where I thought there might be an old GYN or GU or sinus involvement, penicillin or sulfonamides were used. In other words, as a clinician, I used every therapeutic means at my disposal rather than one or more favorites to the exclusion of the others. I therefore urge for this group of disabling rheumatic diseases, a foundation of vitamin E and nutritional therapy, and thereafter all other means be applied to get relief. The use of vitamin E as an antioxidant and sparer of liver damage in carbon tetrachloride toxicity, should be encouraging to those who prefer gold therapy and wish to reduce toxicity.

In turning to the paper by Dr. Steinberg, I recall that, in 1941, I discussed his presentation of fibrositis at the New York State Medical Society convention, where he showed that primary fibrositis was a metabolic disease. I had similar results then, but with accumulated experience I can state boldly that the secondary fibrositic and myositic involvements do just as well with vitamin E therapy as primary ones. It was then that I proposed the theory that vitamin E is a connective tissue metabolite and that it is necessary to the normal physiology of connective tissues. Most recent histological studies and the slides shown today by Dr. Steinberg indicate that the lymphocytic infiltration is in the connective tissues and not in the muscle proper. My theory was then based purely on clinical observations and deductions. Dr. Wechsler had then claimed that the rat's pyramidal tract was injured in vitamin E deficiency. It was soon evident to me that this was erroneous, because the rat has no true pyramidal tract. Therefore, the injury could not be to the nerve tissue, but indirectly to the contracture of connective tissues and its secondary pressure effects upon nerve tissues. When improvement occurred the changes were mostly in the connective tissues. Similarly, fragmentation of muscle fibers in traumatic states or vitamin E deficiencies also returned to normal when the fibrous connective tissues resumed normal functions. It is my conviction that it is insufficient connective tissue formation at the site of the placental implantation that causes abortion in vitamin E deficient rats, and has nothing to do with the fertilization of the ovum or impregnation. The normal activity of vitamin E, therefore, is to maintain normal connective tissue metabolism by acting as a normalizer for hydration by preventing fluid accumulation in local areas that may have either a high acid or high alkaline surrounding, causing first swelling of the fibrous connective tissue contents and later fragmentation of

adjoining muscle fibers. I also maintain that connective tissue is a repair tissue and is in the first line to restore injuries, as evidenced by the increase in endothelial cells, wandering cells or fibroblasts at every site of injury. Vitamin E is a necessary metabolite and stimulant in such repair. A deficiency of vitamin E would interfere with normal repair, leaving scar tissue in a state of construction, tautness rather than normal function. This applies to visible as well as invisible tissue repair.

Another function of vitamin E in connective tissue metabolism is to act as a barrier against infection by being an antagonist to the surface activity of hyaluronidase which may be responsible for the penetration of harmful bacterial or toxic substances. That a working theory is important in clinical research is illustrated by the following incident: A few years ago in discussing the value of vitamin E before the Bethel Hospital Medical Conference, I referred to my belief that vitamin E is a connective tissue metabolite, and that vitamin E is to connective tissue, what vitamin A is to epithelial tissue. The next day a physician called me and asked whether Peyronie's disease could fall into such a disturbed connective tissue classification. He further mentioned that one of his patients is about to undergo an operation for Peyronie's disease and that the urologist as well as he, hold out little hope for relief. When the urologist heard of my suggestion to precede the operation by vitamin E therapy as a groundwork for better healing and possibly less scarring, he placed me in the category of a faddist, to say the least. This afternoon we heard Dr. Scardino speak of treating a group of Peyronie's disease cases with tocopherols with excellent results, and of the observation that some of these patients also suffered from Dupuytren's contraction. All this fits into the theory of tocopherols being necessary for connective tissue metabolism. It is my opinion that this work by Drs. Scott and Scardino should be encouraged as it has a direct bearing on the larger picture of formation of scar tissue.

Let me take this opportunity to congratulate Dr. Steinberg upon his perseverance in applying vitamin E in the clinical entities of connective tissue dyscrasias and for his stimulating influence on the work of Drs. Scott and Scardino. My own work, begun independently and extending over the same length of time, parallels his observations of the value of vitamin E in primary fibrositic conditions. In today's paper, I favor the administration of vitamin E in all chronic rheumatic and arthritic diseases as a foundation therapy regardless of any other adjunctive therapy the physician desires to use.

CLINICAL AND EXPERIMENTAL STUDIES ON VITAMIN E

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The metabolic lesions that precede clinical and histological symptomatology fundamentally consist of a prolonged hyperglycemia, demonstrable with the glucose tolerance test and caused by a retarded utilization of glucose in the muscle fiber (Morgulis), hypercholesterolemia (Morgulis and Spencer), marked increase in oxygen consumption of the muscle (Friedman and Mattill; Aloisi and Meldolesi), and hypercreatinuria and decrease in the muscle creatine phosphate (Verzar, Telford, Emerson, Evans, Goettsch and Pappenheimer, Mackenzie and McCollum, Shimotori, Goettsch and Brown, Knowlton and Hines, *etc.*).

Much attention was devoted by many researchers to the study of the creatinuria and, with brilliant research on nutritional muscular dystrophy of the rabbit, it was established that the creatinuria is a specific symptom of avitaminosis E (Mackenzie & McCollum). Creatinuria precedes every clinical manifestation and, as the syndrome becomes more apparent, the creatinuria increases. The disappearance of creatinuria is considered the most effective proof that the therapeutic element contains Vitamin E. This concept is not accepted by all, however. Morgulis and Spencer do not see any modifications of creatinuria in the rabbit on an E-deficient diet, and Verzar finds hypercreatinuria when paralytic symptomatology is clear and irreversible by alpha-tocopherol treatment.

Creatinuria also appears in physiological conditions, but an increase is found in altered muscle biochemistry due to the break in equilibrium between hydrolysis and synthesis of creatine phosphate, with the liberation of creatine and complete non-utilization by the muscle cell. It is, therefore, a symptom of the lack of muscular efficiency that may have a multiple etiology but which always remains subject to a lack of synthesis of creatine phosphate. For the realization of such synthesis, it is necessary that energy be furnished by the oxidation of carbohydrates. This is demonstrated by the diminution of muscle glycogen, and only the muscle glycogen determines this creatinuria (Brentano).

Numerous experiments were necessary to establish whether this hypercreatinuria is specific for avitaminosis E or whether creatinuria appears in other avitaminoses. Also, much work was necessary to establish the metabolic relationship among creatine, carbohydrates, and vitamin E in the healthy subject.

I have tried to solve the problem by experimenting on:

- (1) Creatine metabolism in different experimental avitaminoses (A, B-1 plus C, and A plus E). A study was carried out periodically (after 3, 6, 9, 12, and 15 months) of an E-deficient diet.
- (2) The modifications which the various vitamin treatments (A, B-1, B-6, niacin, B-Complex, C, and E) determine in the physiological creatinuria of the albino rat and on creatinuria from pre-avitaminosis E.

- (3) The modifications that vitamin E determines on creatinuria from avitaminosis A, B-1, and C.

The results of these experiments (partially completed in 1943, not published before 1945, due to the war, and still partially unpublished) have clearly shown that hypercreatinuria is an unspecific symptom, in that it is found in all the avitaminoses studied, and is the first pathological manifestation which is evident, prior to any loss in the body weight. It can definitely be corrected only by adding the specific vitamin deficient in the diet.

With regard to the specific behavior of hypercreatinuria in avitaminosis E and its tendency to be modified by alpha-tocopherol, the opposing statements of MacKenzie & McCollum and of Verzar may be conciliated as follows: vitamin E cures creatinuria prior to symptomology of vitamin E deficiency when it is the consequence of a metabolic muscular disorder; on the other hand, it is inactive or almost inactive when creatinuria is sustained by the progressive and irreversible destruction of the muscle cell (after the 12th month of a vitamin-deficient diet in the rat).

Interesting data, from the pathogenic point of view, were furnished by the study of the modifications of the physiological creatinuria of the rat and of the guinea pig with vitamin treatments (A, B-1, B-2, B-6, niacin, B-Complex, C and E): vitamin E causes a constant and prolonged disappearance of the normal urinary creatine, small as it is; vitamin A is completely inactive; and vitamins B-1, B-2, B-6, niacin, B-Complex, and C reduce the urinary excretion of the creatine for only a short period of time.

I had important results with vitamin E on creatinuria of avitaminosis A, B-1, C, and respectively with vitamins A, B-1, and C on creatinuria of preavitaminosis E. Alpha-tocopherol acetate greatly retards the formation of hypercreatinuria of avitaminosis A and only partially of avitaminosis B-1; hypercreatinuria of preavitaminosis E is only slightly diminished by treatment with vitamins B-1 and B-2. Vitamins A, B-6, niacin, B-Complex, and C appear to be totally inactive.

Vitamin E, therefore, shows accelerated activity which normalizes the creatine metabolism in the normal animal as well as in experimental avitaminosis (A, B-1). Thus it acts as a regulator of the muscular biochemical process, stimulating better nutrition in the muscle cell. It fails to produce any effect however, when histological changes take the place of functional lesions. Similar activity of a minor type is possessed by vitamins B-1, B-2, niacin, and C. Creatinuria, therefore, can be considered neither a specific symptom nor a primary symptom caused by a vitamin E deficiency alone, but is an expression of altered muscle metabolism, evidenced through the increased excretion of creatine.

Brentano demonstrated that the metabolism of creatine is tied in with the metabolism of carbohydrates. Therefore, I studied the relationship between vitamin E and carbohydrate exchange with the following methods: (1) changes in both fasting blood sugar and glucose tolerance curves of arterial and venous blood in the human being and changes in the glycogen reserve in guinea pigs, after administration of alpha-tocopherol; (2) changes in creatinuria caused by toxic substances (diphtheritic toxins), endocrine

I arrived at the logical corollary of making a therapeutic attempt to treat diabetes mellitus with vitamin E. Here, the arteriovenous differential glycemia is practically zero, due to the lack of utilization of the carbohydrates by the muscular cell. My results are in full agreement with my theoretical premises. The treatment of diabetes mellitus with vitamin E, initiated in 1942, increases the differential between arterial and venous glycemia and reduces the hyperglycemia and glycosuria. In a mild form of diabetes, the metabolic changes may be cured with the use of vitamin E alone. In a severe case of diabetes, in which the specific treatment cannot be discontinued, alpha-tocopherol acetate increases the intensity and duration of the hypoglycemic effect of insulin. The more complete utilization of carbohydrates in diabetes is demonstrated by the disappearance of glycosuria, when the glycemic values are still over the usual renal threshold. Vitamin E has no action on the renal level limit so far as glucose is concerned. I have reached this conclusion as a result of my research in a case of renal diabetes (FIGURES 3-5).

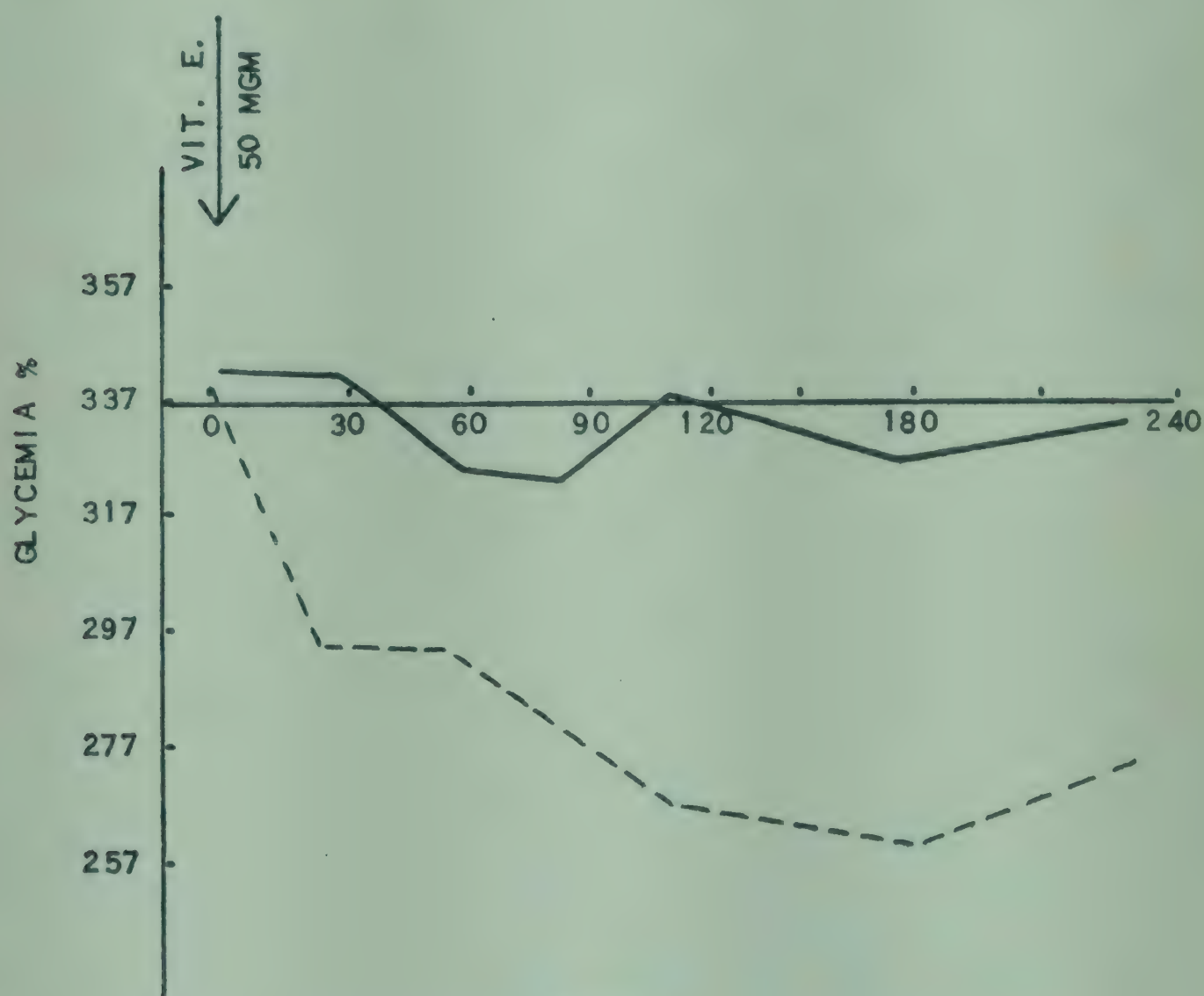


FIGURE 3.

I have, however, treated approximately 70 cases of diabetes mellitus. In four or five cases of severe diabetes, no beneficial results were obtained. One failure on autopsy showed a pancreatic calculus so large that the entire pancreas was fibrotic and the liver almost completely degenerated. Another had become insulin resistant, but no help was afforded by the E and the patient died in coma. Approximately 40 cases of severe diabetes with blood

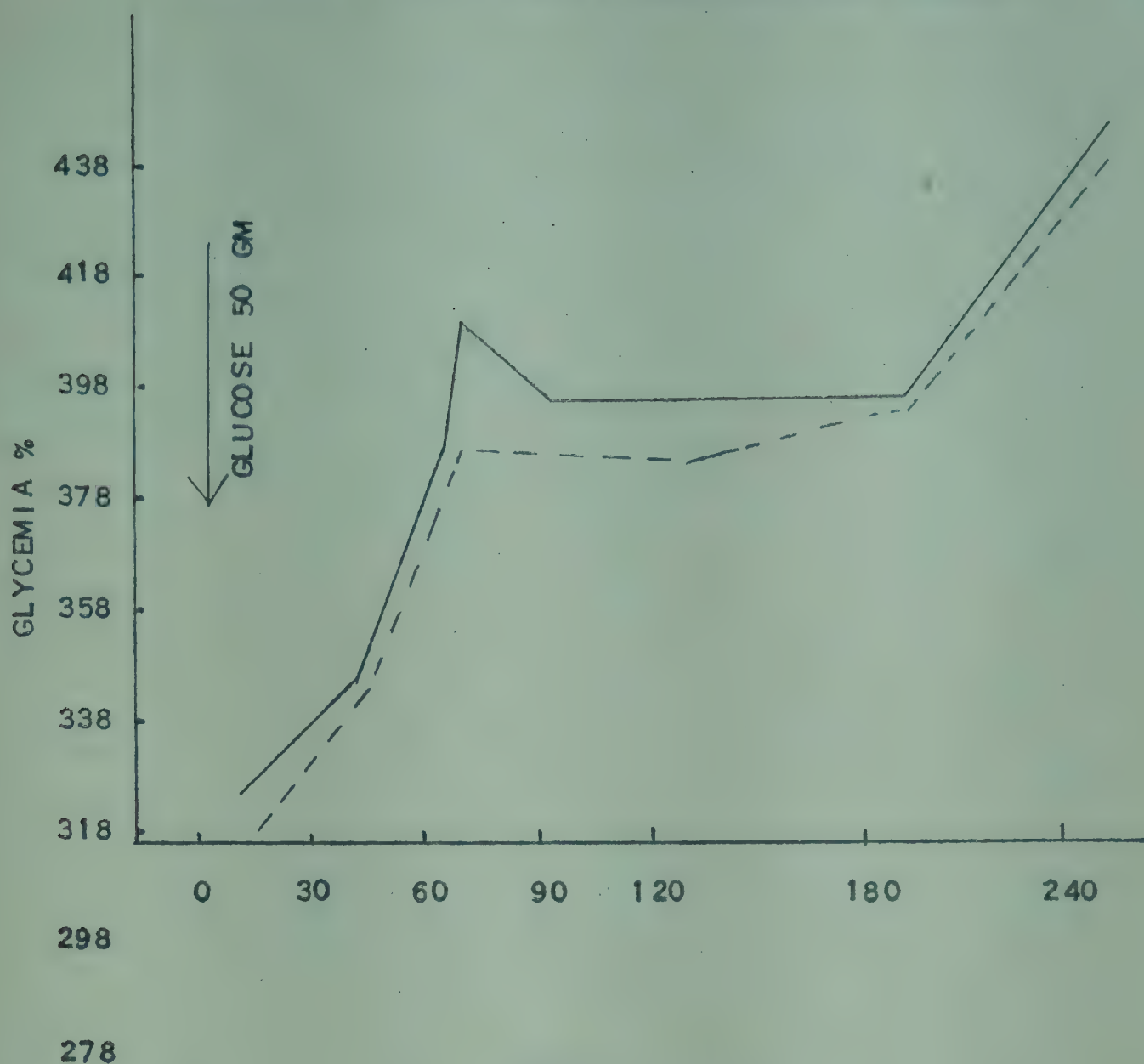


FIGURE 4.

sugar above 280 and exhibiting ketonuria were controlled much more readily with insulin plus E than a parallel group treated with insulin alone. Twenty-five cases of moderate diabetes, with blood sugars less than 220, were brought to normal with E alone. Nine cases with E alone did not return to normal. Insulin was added for five days to control them and then they remained under control on E alone. These results have been confirmed by Cataldi e Volpe.

The second group of experiments on the relation of creatinuria to thyroxin (FIGURE 6) and diphtheric toxine (TABLE 1) showed that vitamin E maintains creatine metabolism within normal limits in the presence of endocrine and toxic intoxication.

A study, completed though still unedited, has been conducted with Dr. Ubaldo Arduini on the changes in the blood and urinary levels of creatine and idiopathic muscle edema in persons affected by serious general diseases. This muscular edema is an expression of pathological exchange of creatine in the muscle (Leitinger) and, according to our experience, begins only when creatinuria surpasses 100 milligrams daily. Prolonged treatment with alpha-tocopherol leads to the progressive disappearance of creatinuria and to the reduction of muscle fibrillation, although the course of the basic disease remains unchanged. When the treatment is discontinued, creatinuria slowly reaches the previous level again.

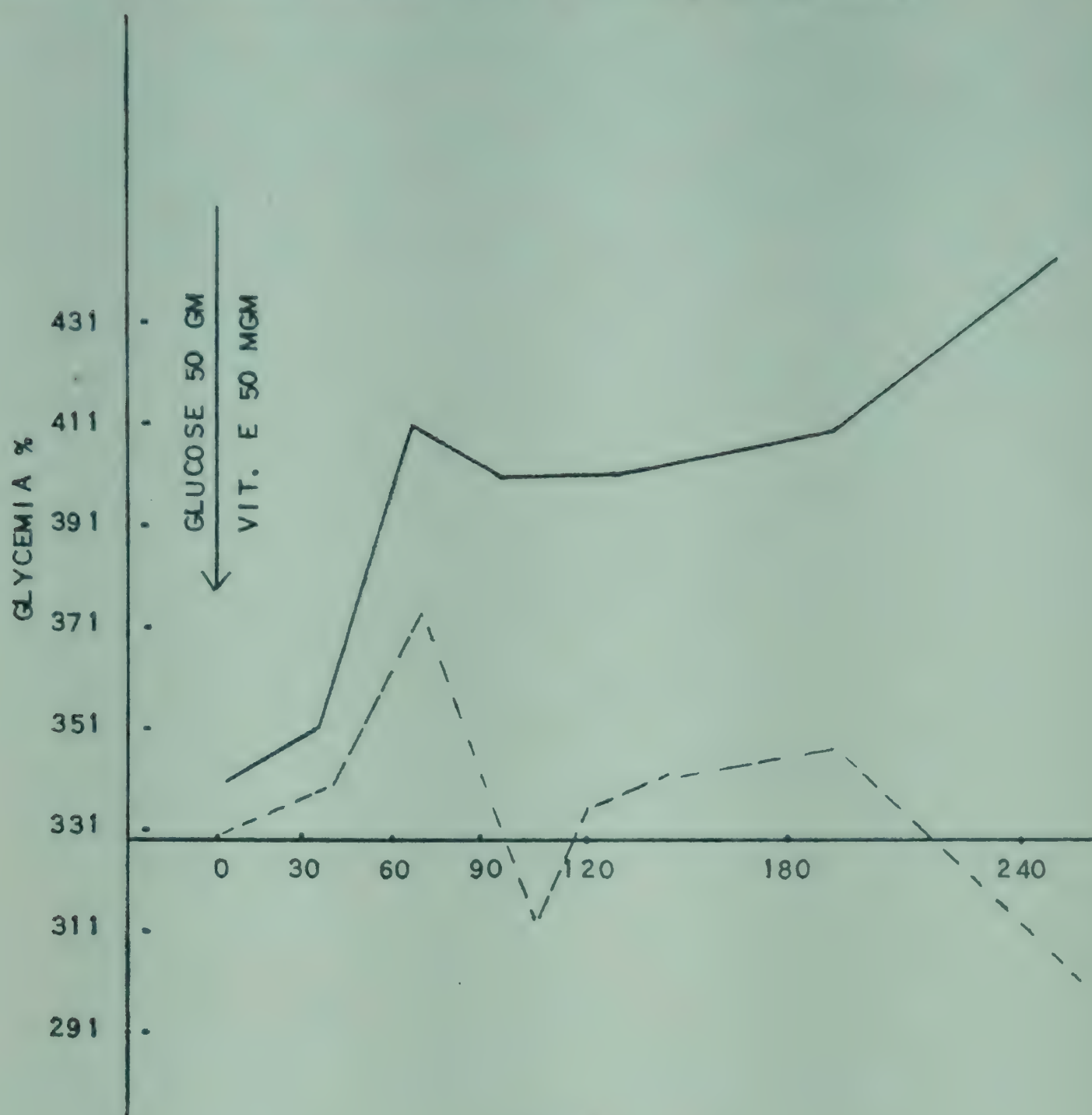


FIGURE 5.

The same results are obtained with combined treatment with glucose plus insulin. The cases studied had normal blood sugar. Their muscles were suffering from lack of glycogen. Therefore, their contraction function was carried out abnormally, with a lack of resynthesis of creatine phosphate and consequent creatinuria. This creatinuria is comparable in its pathogenesis to the diabetic form which begins simultaneously with the acidosis and ketonuria caused by poor glucose utilization (Colangili and Breda).

The second group of experiments shows—if we take into account the importance of creatinuria as a diagnostic element for the function of the muscle cell—that the nutritional changes in the muscle affected by thyroxine, diphtheric toxine, and general dyscrasias were almost normal after treatment with vitamin E. Vitamin E facilitates glucose utilization in the muscle cell.

Creatine is found in all muscles, voluntary as well as involuntary. For this reason, I do not agree with Morgulis and Olcott, according to whom lesions deriving from vitamin E deficiency are to be found only in the voluntary muscles.

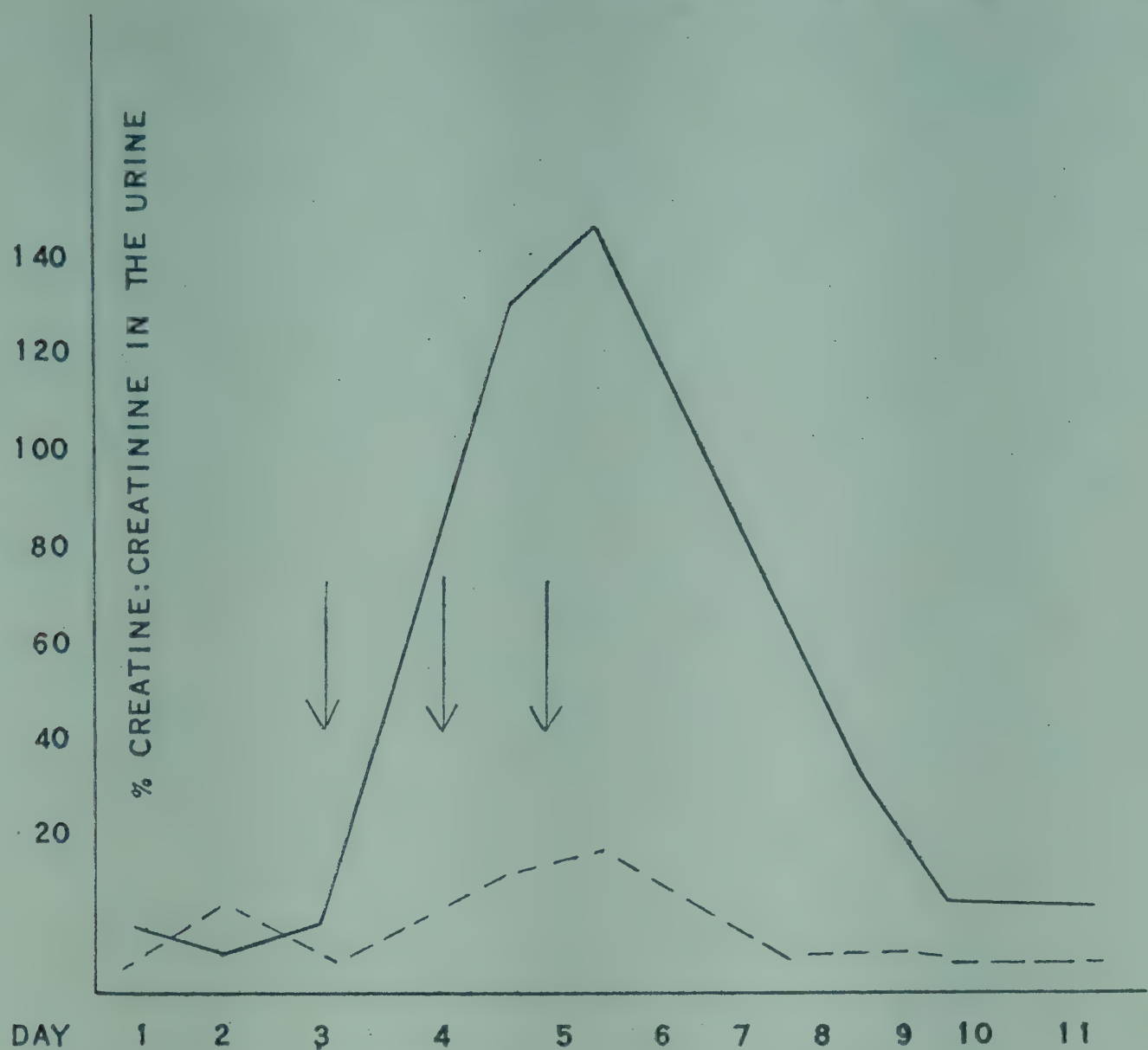


FIGURE 6. ———— Thyroxin: 0.2 mg. daily.
----- Thyroxin: 0.2 mg. daily, plus 50 mg. alpha-tocopherol.
↓ Treatment.

The histological examination of the hearts of rats in avitaminosis E indicated also that the myocardium presents degenerative lesions, taking place around the ninth month of E deficiency and becoming more and more progressive, evident, and widespread. As to this point, I agree with Bird

TABLE 1
DIPHTERYTOXIN ($\frac{1}{4}$ D.L.M.)

Group	Vitamin E mg.				% Creatine: Creatinine in the urine			
	Treatment				Data			
	Preven.		Curative		7/×11	29/×11	9/1	9/11
	mg.	day	mg.	day				
Negat. contr.	—	—	—	—	4.6	42.7	67.0	46.4
Curat. treatm.	—	—	40	10	7.5	12.3	24.7	15.3
			10	53				
Prev. A.	10	6	40	10	5.5	8.5	12.2	7.3
Curat. treatm.			10	53				

and Cultom, MacKenzie, Mattill, and others. The muscles of the uterus also present profound degenerative phenomena with fibrosis (Demole, Mason, *etc.*).

Therefore, I believe that vitamin E is an indispensable and specific substance for the normal nutrition of the muscle. Its deficiency determines the degenerative lesions, which are well known. It enters into the metabolism of muscles by an extremely complex mechanism in an oxidation-reduction system but also (perhaps mainly) as an intervening factor in the phosphorylation of creatine in carbohydrate metabolism. The lack of utilization of carbohydrates on the part of the muscle leads to metabolic changes that begin with creatinuria and cause, at a later stage, definite degeneration of the muscle itself. This degeneration is reversible with vitamin therapy up to a certain point but becomes irreversible when connective tissue is substituted for the functioning parenchyma.

The muscle lesions resulting from a pathological process in the nutrition of the muscle cells are sufficient to explain habitual abortion as well as the paralytic syndrome and the death of the deficient animal. I am definitely against the concept of habitual abortion being caused by hormonal lesions, when, in E deficiency, the cycle of estrus and the evolution and fecundability of the ovum are normal (Evans) and there exists an extensive degeneration of the muscle masses of the fetus (Goettsch; Pappenheimer; and Aloisi) and of the uterine fiber cells filled with pigments.

What I have found, since 1945, on the basis of research on the metabolism of creatine and carbohydrates carried out through histological examination of the muscular system in E-deficient animals (albino rats), has been amply confirmed by Luttrell and Mason, Malamud, Nelson, Evans, and others. They consider that the degenerative neural lesions come after the muscular lesions. In rats, the muscular lesions precede the neural lesions by about two months. The same may be said for endocrine lesions which can be explained as a result of acidosis from deficient metabolism, which the author and others have studied.

Clinical application of experimental research may be seen in the treatment of not only diabetes but also diphtheric toxicosis and postdiphtheritic paralysis. Favorable results have been obtained in both preventive and curative aspects.

As a clinical-therapeutic corollary of these experiments, I take the liberty to say a few words about the therapeutic use of vitamin E in the human neuromuscular syndrome. The practical solution of this problem is still under discussion. On one side are the praiseworthy and considerable successes obtained in cases of progressive muscular dystrophy and amyotrophic lateral sclerosis by Stone, Bicknell, Wilkinson, Marcel, Bang, Einarson, Fog and Ringsted, Wechsler, Rosenberg, Kirstein, Monnier, and others; and, on the other side, are the completely negative results, not only on the course of the disease but also on the creatinuria which accompanies it, according to Denker and Scheinmann, Ferrebee, Klingman and Franz, Hager, Byrne and Baker, Churschmann, Harris, Alpers and others.

Following Moore's and Ottonello's theory, that amyotrophic lateral scler-

rosis (to cite one simple example) is the consequence of whatever toxic or infectious agent operates on a nervous system chronically deficient in nicotinic acid, it is illogical—and this is also confirmed by my personal experience—to expect any clinical or symptomatological results from treatment with alpha-tocopherol, since, in this case, the disease might be sustained by another vitamin deficiency. The eventual disappearance or decrease of creatinuria should be attributed to better muscular nutrition and not be considered diagnostic of vitamin deficiency. A thorough statistical study of myo- and myelopathic diseases confirms the results of my experiments on E-deficient rats. The best results were obtained when the disease was at its primary stage. The results were negative in patients suffering from disease at an advanced stage.

In conclusion, I wish to point out that, before denying or affirming the therapeutic action of any vitamin in neuro-muscular diseases in the human being, it is advisable to stress the following two points: (1) it is necessary to ascertain which vitamin deficiency is directly or indirectly responsible for the disease; (2) it is important to find out whether the lesions are already irreversible, even if vitamin therapy is to be used only as a helpful symptomatic therapy.

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VITAMIN E IN THE TREATMENT OF DIABETES MELLITUS*

By Arthur Vogelsang

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The author has treated cardiovascular diseases since September, 1945, with alpha-tocopherol and has noted in previous publications^{1, 2} some of the effects obtained in the diabetic patients on whom this therapy was used.

The purpose of this paper is to present certain general statements on the results of over two years experience in the use of alpha-tocopherol on diabetic patients.

Method

The method usually employed consists of the following steps:

(a) The patient is rigidly held on a definite diet—sufficient for the exertion required of him and adequate to maintain his weight. The diets used are those of Baltzan.³

(b) The diabetes is controlled with protamine zinc insulin, alone or in combination with the regular insulin, in a dose sufficient to provide a fasting morning glycosuria ranging from a trace to one per cent. One capsule Squibb's "Special Vitamin Formula" daily provides the minimal daily requirement of vitamins A, B-Complex, C, and D.

(c) The patient supplements periodic glucose tolerance tests and other blood sugar estimations with a daily urinalysis before breakfast and also, in some cases, by a specimen at 5 P.M. He uses the Sheftel method, as in the urine test case of the Eli Lilly Co. This has proved to be a most dependable method.

(d) When the diabetes is controlled as in the above steps, the patient is given Vascuals alpha-tocopherol (V.C.A.—100 mg. per capsule), one capsule three times a day before meals, making a total dose of 300 mg. alpha-tocopherol daily.

It is obvious that, when a patient is first seen in a diabetic emergency, such as gangrene, the above method cannot be instituted in a leisurely manner but must be modified to obtain relief as rapidly as possible. In such cases, 200 to 400 mg. per day of alpha-tocopherol, injected intramuscularly for the first two to five days, is efficacious, varying this of course according to specific features of the individual case.

To avoid destruction of the oral tocopherol, no inorganic iron preparations are administered at any time.

Observations

As case histories were published previously¹ and read at the Montreal Symposium, they will be omitted here. The following general statements cover observations noted with the treatments already described:

(a) No change was seen in the blood-sugar levels of non-diabetic patients.

(b) All diabetics over 25 years of age showed a marked decrease in insulin requirement within two months. Practically all were able to abandon the use of insulin after one year of E therapy.

* This is the only publication by The New York Academy of Sciences of this paper. Any prior publication thereof was without the Academy's consent.

(c) This therapy, in no case, aggravated the condition.

(d) The period of time under treatment before an effect was noted varied greatly with different patients.

(e) No cases became comatose.

(f) Insulin reactions were noted in many cases. They were, however, quite mild in character and consisted only of a sensation of "floating" or giddiness. These symptoms were relieved if the patient merely paused to sit or recline. The quickest way to speed recovery was to have the patient take two tablespoonsful of whisky, an equal amount of hot water and half a teaspoon of sugar well stirred. If no materials were at hand however, the patient felt better after a short rest. No serious reactions, with loss of consciousness, *etc.*, were noted. This may be accounted for by the fact that the E-treated diabetic has accumulated stores of reserve muscle and liver glycogen⁴ which the ordinary diabetic does not possess. It is these reserve stores that are mobilized to relieve the hypoglycemia.

(g) With the few juvenile diabetics treated, the results, as far as insulin reduction is concerned, were not so marked. A third required less insulin. The most prominent effect on these cases was the improved rate of growth and the increased hardiness of the children, whether they required as much insulin or not.

One boy of nine, who had been a frail little lad, not only subjugated the rest of the children in his area but actually advanced from the "foot" of the class to the "head" in four months—much to the surprise of his teacher and his parents. It is too early to generalize regarding the effect on diabetic children, but the author is of the opinion that continued administration over several years should materially help these unfortunate youngsters.

(h) Cases complicated by gangrene or perforating ulcers demonstrated an increased redness, warmth, and more prominent tissue reactions about the affected area. This began after four or five days and continued until the lesion was healed.

Even in cases of complete arterial occlusion, where no pulsations were evident upon the oscillometer, warmth and redness of the affected extremity became evident in about four days, although it may have been from three weeks to two months before arterial pulsations were detected. As in E therapy of Buerger's disease, sensations of warmth, prickling, or even severe pain were noted by some patients during the first two weeks of therapy or even longer.

(i) The fall in glycosuria and glycemia on E therapy was not a steady process. Flat plateaus were broken every so often by sharp declines in the glucose levels.

(j) Those patients who, for reasons of their own, discontinued the alpha-tocopherol found that increasing glycosuria and blood sugar levels returned gradually in from five days to one month. On resuming treatment, the condition again improved.

Discussion

The mode of action of alpha-tocopherol on diabetes in adults is not definitely known. In a previous paper,¹ the author suggested that, as it has been shown that E improves the blood supply to skin and subcutaneous

tissues,⁵ heart muscle,⁶ aberrant collagenous tissues,⁷ etc., the effect on diabetes could be explained on a basis of improved circulation to the islets of Langerhans. Butturini, however, on the basis of excellent experimental work, advances the idea that this effect can be explained by improved utilization of glucose by the muscle cell. His work also implies that phosphorylation of glucose in the liver might be aided by E, so that larger reserves of liver and muscle glycogen can be accumulated. It may be that both of these explanations apply, although the clinical experience of the author tends to support the conclusions of Butturini.

Even if the insulin requirements were not affected, the use of E in diabetes would be justified by the success it has demonstrated in treating the cardiovascular complications.

Whatever the mechanism of the action of alpha-tocopherol may be, the constancy of its effect on diabetes indicates that it should be administered, in proper dosage, to every diabetic patient.

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Discussion of the Paper

DOCTOR E. H. BENSLEY (*Department of Metabolism and Toxicology, Montreal General Hospital, Montreal, Canada*): A study of the therapeutic value of oral administration of mixed tocopherols in diabetes mellitus is being made by the Department of Metabolism and Toxicology and the Pharmacy of the Montreal General Hospital. This study was started in November, 1948, and is not yet complete. Final and detailed analysis of our results will not be made until after June 1949, but sufficient work has been done to justify a preliminary statement.

Capsules containing mixed tocopherols (Natopherol Abbott and Natopherol acetate Abbott) and matching placebos have been donated for this study by Abbott Laboratories Limited. Thirty-three patients are receiving mixed tocopherols; twenty are receiving placebos. All cases are adult diabetics attending the Out Door Clinic of the Montreal General Hospital. Doses of tocopherols range between 100 I.U. vitamin E (110 mg. mixed d-tocopherols) and 600 I.U. vitamin E (660 mg. mixed d-tocopherols). Duration of tocopherol administration in individual cases is now 5 weeks to 4½ months. No beneficial effects of tocopherol therapy on control of diabetes have been detected.

THE EFFECT OF VITAMIN E UPON SPERMATOGENESIS

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Four preparations of vitamin E were administered to a series of 19 relatively fertile and subfertile men, with the object of increasing the number of their sperm cells.

Over 100 semen analyses were performed, for control (before treatment) and test (during and after treatment) purposes. The individuals received minimum total dosages of 3000 milligrams of material during the interval of 24 to 90 days.

The analyses were based on the method of the author.¹ They included a consideration of the following characteristics as listed in TABLE 1: volume of the ejaculate in cc.; number of active and inactive sperm per cc. (millions); number of active and inactive sperm in total ejaculate (absolute motility) in millions; percentage of active sperm (motility); speed of sperm (in seconds); percentage of specimens showing active sperm at the end of 24 hours; and the percentage of oval forms.

A basis for the classification of male fertility was described previously.² Using the absolute motility figure (the total number of moving sperm per cc. multiplied by the volume of the ejaculate) as the unit of measurement, the men were classified as either relatively fertile (80 to 185 million active sperm) or subfertile (below 80 million active sperm).

Effects of Vitamin E Therapy

TABLE 1 shows the effect of the vitamin E on the semen. Four preparations of vitamin E were administered: (1) mixed tocopherol; (2) alpha-tocopheryl phosphate; (3) ephynal acetate; and (4) delta-tocopherol. The table records counts made before and after treatment with each of the four vitamin E preparations. Under "Experimental conditions" are listed the number of days the treatments were performed, the total number of counts, and the range of dosage.

The treatments produced no significant change in the semen picture. The slight increase in the number of active sperm in the total ejaculate (line 7), following the administration of ephynal acetate, is within the normal range of variation for an individual.

From the present observations, it is concluded that vitamin E does not stimulate spermatogenesis.

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* This investigation was aided by grants from the Committee on Therapeutic Research, Council on Pharmacy and Chemistry of the American Medical Association, and the Samuel S. Fels Fund.

TABLE 1
SUMMARY: OBSERVATIONS BEFORE, DURING, AND FOLLOWING TREATMENT WITH VITAMIN E

	1		2		3		4	
	Mixed tocopherol		Alpha-tocopherol phosphate		Ephynal acetate		Delta-tocopherol	
	controls	24-90 da.	controls	30 da.	Controls	30 da.	controls	30 da.
1. Experimental condition*	27 counts	40 counts 6000-36,000 mg.	6 counts	8 counts 3000 mg.	4 counts	5 counts 3000 mg.	5 counts	7 counts 4500 mg.
2. Number of individuals	10	10	3	3	2	2	4	4
3. Volume† of ejaculate (cc.)	3.4 (1.4-5.6)	3.5 (1.4-6.0)	3.0 (1.4-5.6)	3.7 (2.0-6.0)	2.8 (1.8-4.6)	3.0 (2.1-4.4)	4.2 (2.0-7.4)	4.3 (2.4-8.0)
4. Active and inactive sperm per cc. (millions)	49 (10-186)	41 (4-129)	41 (6-105)	32 (4-84)	59 (29-82)	82 (22-198)	34 (6-88)	35 (12-58)
5. Active sperm per cc. (millions)	15 (2-43)	12 (2-44)	13 (2-31)	9 (2-20)	24 (10-30)	25 (22-198)	14 (3-34)	12 (5-26)
6. Active and inactive sperm in total ejaculate (millions)	142 (26-375)	113 (23-309)	89 (13-210)	115 (23-352)	149 (116-186)	177 (66-255)	101 (17-166)	116 (34-226)
7. Active sperm in total ejaculate (absolute motility) (millions)	43 (3-111)	38 (5-103)	28 (5-63)	31 (6-77)	59 (39-79)	75 (33-125)	40 (7-68)	39 (12-58)
8. Percentage of active sperm (motility)	32 (11-60)	32 (8-55)	33 (23-54)	31 (21-52)	39 (34-54)	42 (34-50)	35 (22-56)	41 (25-67)
9. Speed (drive) of sperm (seconds)	1.5 (osc.‡-2.4)	1.3 (.9-2.1)	1.2 (1.0-1.3)	1.2 (.9-1.5)	1.2 (1.1-1.2)	1.1 (1.0-1.2)	1.2 (1.1-1.4)	1.2 (1.0-1.4)
10. Percentage of specimens active after 24 hours	few-0	few-0	—	—	few	few	few-0	few-0
11. Percentage of oval forms (Stained specimens)	78 (76-88)	74 (30-91)	—	—	64 (61-67)	83 (74-83)	73 (37-90)	75 (59-82)

* The first emission was always preceded by five days of abstinence.
† The values are averages for the specified number of individuals. () Ranges.
‡ Oscillating.
Note in line 7 that the numbers of active sperm in the total ejaculate show practically no changes following the treatment with the four vitamin E preparations.

A TRIAL OF VITAMIN E THERAPY IN JUVENILE DIABETES MELLITUS

By George M. Guest

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Thirteen patients attending the Cincinnati Children's Hospital Diabetic Clinic were given Vitamin E during periods of from 50 to 100 days without discernible effect on their requirements for insulin.

The patients ranged in age from 8 to 17 years, 6 girls and 7 boys, with duration of diabetes from 2 to 9 years. They were selected at random from among 50 patients attending the clinic. All follow a so-called "unrestricted" dietary regime with the insulin dosage adjusted to allow constant mild glycosuria, but with constant attention to the avoidance of acetonuria.^{1, 2} The patients keep a daily record of tests (qualitative) for sugar and acetone in the urine, of the daily insulin dosage, and of insulin reactions or other untoward symptoms.

In most instances, the insulin employed was a mixture of 2 parts protamine zinc insulin to 1 part regular insulin, injected as a single dose in the morning. The average total daily dose in the group of 13 patients was 55 units, varying from 20 units, minimal, to 120 units, maximal.

Three thousand capsules,* each containing 75 mg. of d, alpha-tocopherol acetate were distributed to the patients in lots of 150 to 300, with instructions to take 3 capsules a day until the supply was exhausted. At their next regular visits to the clinic, after the usual two to three months intervals, no changes were noted in the pattern of the records of insulin dosage or glycosuria, either during the period of taking the capsules or after the supply was exhausted. One boy came into the hospital with mild acidosis during the period when he was taking the Vitamin E capsules, an episode ascribable to an acute infection and his failure to increase the dose of insulin (according to established custom) as needed at the onset of this infection. Before the episode, he was taking 85 units of insulin daily. After recovery and return home (5 days hospitalization), he has continued taking 100 units of insulin a day until the present time.

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* Supplied by Doctor Philip L. Harris, of the Distillation Products, Inc., Rochester, N. Y.

SOME EXPERIENCES WITH THE USE OF VITAMIN E IN VARIOUS CARDIAC CONDITIONS

By Samuel Baer, William I. Heine, and D. Barton Gelfond

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Sometime ago, we decided to evaluate the therapeutic results obtained in the treatment of various cardiac disturbances with vitamin E. Patients were selected with angina pectoris, hypertensive and/or arteriosclerotic heart disease, and cardiac failure having as its cause one of a number of etiological conditions. Following a preliminary survey that included physical examination, electrocardiogram, and orthodiagram, these patients were begun on 300–400 mg. of vitamin E daily (by mouth). This plan of therapy was continued for 3–6 months; examinations, cardiograms, and orthodiagrams were repeated frequently during the study.

Before attributing improvement to an agent used in the treatment of any cardiac condition, we felt the following criteria should be met:

(a) In angina pectoris, we are frequently forced to measure improvement by symptomatic response. This is always open to criticism and errors in interpretation. Wherever possible objective estimations of improvement are preferable. In addition, if a placebo is unknowingly substituted for the therapeutic agent supposedly producing improvement, there should be a prompt recurrence of symptoms.

(b) In hypertensive or arteriosclerotic heart disease, a therapeutic agent should produce fall in blood pressure, improvement in dyspnea, reversal of electrocardiographic abnormalities, or decrease in cardiac enlargement that is radiologically demonstrable.

(c) In cardiac failure (whatever its etiology), a drug meriting consideration should produce decrease in pulmonary, hepatic, abdominal, or peripheral edema. It should slow the ventricular rate in auricular fibrillation. If the patient has been restored to compensation with digitalis and/or mercurials, the new therapeutic agent should prevent the return of cardiac failure if these preparations are withheld.

The natural life history of the disease must always be borne in mind. We should hesitate to attribute to a drug, improvement that is a part of the normal recovery pattern in the illness in question.

With these criteria in mind, we studied the effect of vitamin E in 30 patients with organic heart disease. In no case was there demonstrable decrease in cardiac failure. In no patient could we demonstrate electrocardiographic improvement or orthodiagraphic decrease in cardiac size. Even in the few patients with angina pectoris presenting questionable improvement in symptoms, we did not see any patient reporting improvement that could not be duplicated by a placebo. We therefore were forced to conclude that, in our hands, vitamin E had nothing to recommend it as a therapeutic agent in heart disease.

VITAMIN E (ALPHA-TOCOPHEROL) IN TREATMENT OF THROMBOANGIITIS OBLITERANS AND LEG ULCERS*

By L. Lewis Pennock

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Various reports have appeared on the favorable effect of vitamin E (alpha-tocopherol) in peripheral vascular diseases.^{1, 2, 3, 4} The list of diseases includes thrombophlebitis, phlebothrombosis, indolent varicose ulcers, early gangrene, thromboangiitis obliterans, arteriosclerotic gangrene, sclerosis of legs with ulcers, and noduloulcerative granuloma of the legs.

In view of these reports, a series of cases under our personal supervision in a hospital were given a preparation of alpha-tocopherol in varying doses. Oral therapy consisted of 200 to 600 mg. daily. Intramuscular therapy varied from 200 to 400 mg. daily. These doses are considered adequate both by the manufacturer and in the published reports.

Clinical Studies

Thromboangiitis Obliterans. Case 1. L. R. G.: a 29 year-old white male with onset, 5 years ago, of intermittent claudication and pains in the soles of his feet. He developed infection and gangrene in his right first toe in 1944 and had an amputation of the terminal phalanx of this toe that year. In 1945, infection and gangrene occurred in the left first toe. Amputation of this toe failed to heal and a below knee amputation was done in 1945. With an artificial limb, he continued to work, driving a truck. In October, 1948, he injured the stump, developed a painful ulcer, and was admitted to the hospital in November, 1948. Despite advice to the contrary, he has persisted in smoking off and on.

Examination showed a deep draining ulcer of the stump. The dorsalis pedis and posterior tibial pulsations were absent in the right foot. The Allen test indicated partial occlusion of the right radial and ulna. He was started on vitamin E, 300 mg., later 600 mg., orally plus 200 mg. intramuscularly daily. Local wet dressings were used. The pain became increasingly severe and could not be controlled with demerol, barbiturates, or nerve block for any length of time. Relief was finally obtained by spinal anesthesia and ice packs. Amputation above the knee was done 10 days after start of tocopherol therapy. Healing was uneventful, without any E medication, but the patient has stopped smoking again.

Case 2. M. Mc. C.: a 54 year-old white male with onset, 10 years ago, of intermittent claudication, ulcers of left first toe, right second toe, and several fingers of both hands. These healed spontaneously. Six weeks before admission, he developed an ulcer of the right second toe which failed to heal. He has never stopped smoking.

Examination showed cyanosis of right second, third, and fourth toes, with an ulcer on second toe. The dorsalis pedis was absent bilaterally.

* The vitamin E was supplied by VCA Laboratories in capsules containing 100 mg. of alpha-tocopherol for oral use and in sesame oil for intramuscular use.

All other pulsations were present. The oscillometric index at the ankle was normal. There was plantar ischemia bilaterally and marked vasospasm.

Treatment consisted of oscillating bed, intravenous typhoid, etamon, priscol, and lumbar blocks. Vitamin E was given, 300 mg. orally and 100 mg. intramuscularly daily. He refused to stop smoking completely. The gangrene progressed, involving the third toe. A right lumbar sympathectomy produced a dry warm leg, but the gangrene progressed. A guillotine amputation of the second and third toes was done 60 days after admission. The vitamin E was increased to 600 mg. orally and 300 mg. intramuscularly daily. The amputation sites healed slowly, but gangrene developed in the fourth toe and guillotine amputation of the fourth and fifth toes was done 30 days later. This healed within a month. He had stopped smoking after his second amputation.

Case 3. R. A. M.: a 38 year-old white male with onset, 2 years ago, of pains in right foot and intermittent claudication. Six months before admission, he developed aparonychia of the right first toe, and it was noted that he had dependent rubor, pallor on elevation, numbness, coldness, and sweating. He was treated with tetrathione, rutin, and papaverine with no results. He continued to smoke. Because the infection progressed and he developed an ulcer at the infection site, he was admitted to the hospital.

Examination confirmed the above, plus absent dorsalis pedis and posterior tibial on the right. All other pulsations were present. The oscillometric readings were normal. The right leg was colder than the left.

Treatment consisted of the oscillating bed, right lumbar sympathetic blocks, and etamon. He gave up smoking completely. He was discharged healed in 60 days. Vitamin E had been ordered, but, through an error, he had only 10 days of therapy, 600 mg. orally daily, just before discharge.

Case 4. D. W. H.: a 32 year-old white male with onset, 5 years ago, of tingling, numbness, and pain in the left foot and intermittent claudication after walking one or two blocks. Three years ago, he developed gangrene in the left first toe and a mid-thigh amputation was done. In December, 1948, a callus on his right first toe was excised, leading to an ulcer which failed to heal. He was admitted to the hospital a month later. He has never stopped smoking.

Examination revealed an ulcer surrounding the tip and lateral surface of the right first toe. The only palpable vessels in either extremity were the femoral. The oscillometric readings in the right leg were markedly diminished. The leg was cold and wet, blanched on elevation, and showed rubor on dependency.

Treatment consisted of the oscillating bed, wet dressings, right lumbar block, etamon, and priscol. Nerve blocks were used to combat pain. Vitamin E was started at 300 mg. and later increased to 600 mg. orally daily, plus 400 mg. intramuscularly daily. The lesion progressed for a time, then became stationary. He gave up smoking after admission to the hospital. Three months after admission he finally consented to right lumbar sympathectomy. The lesion is still not healed, although it is improving.

Case 5. R. F.: a 50 year-old white male with onset, 13 years ago, of pain and weakness of all his fingers, and later toes, with loss of finger nails and spontaneous amputation of distal parts of several fingers and toes. Two weeks before admission, he injured his left foot and developed ulcers of the second and fourth toes. He has never stopped smoking.

Examination showed ulcers on left second and fourth toes—left fifth toe absent (amputated). The dorsalis pedis and posterior tibial pulsations were absent bilaterally. Allen test showed partial occlusion of both ulna arteries. The oscillometric readings were diminished in both ankles and wrists.

Treatment consisted of oscillating bed, etamon, priscol, and wet dressings. Vitamin E was given in doses of 600 mg. daily orally. He refused to stop smoking. The lesions progressed, and the 2nd, 3rd, and 4th toes were amputated by guillotine method 21 days after admission. The amputation sites are now healing.

Case 6. E. W.: a 54 year-old white male with onset, 13 years ago, of intermittent claudication in both legs and repeated attacks of superficial migrating thrombophlebitis. Over these years, he has suffered amputation of both legs above the knees, amputation of first and second fingers of left hand and the right second finger, and partial amputation of the right third and fourth fingers. His present admission was for the ulcer on the left fourth finger and an ulcer on his right thigh stump. He has never stopped smoking. He has a severe superficial thrombophlebitis on his left forearm.

He was treated with wet dressings, local nerve block for pain, etamon, and priscol. In addition he was given vitamin E, 600 mg. orally and 200 mg. intramuscularly daily. The ulcer on the stump has shown no improvement 60 days after admission. The pain continues. The stump ulcer was finally excised. The finger ulcer is progressing slowly.

Arteriosclerosis Obliterans. *Case 7.* C. M.: a 56 year-old white male with intermittent claudication in his left leg for the past year. In the past six months, this had become more severe, limiting his walking to one-half block, and he noted marked redness of his foot. A day before admission he noted pus beneath left first toenail.

Examination showed cold left foot, plantar ischemia and dependent rubor, and an ulcer beneath the left first toenail. The only pulse palpable in either leg was the right femoral. The oscillometric reading was zero in left ankle and calf, $\frac{1}{4}$ in right ankle, and $\frac{1}{2}$ in right calf.

He was placed on vitamin E, 300 mg. orally daily and was placed in an oscillating bed. Etamon and priscol produced no results, nor did surgical sympathetic blocks. The ulcer progressed and became more painful and mid-thigh amputation was done six weeks after admission. Following amputation, vitamin E was increased to 600 mg. daily. Two weeks after amputation, the patient injured the stump. The incision site gaped widely and became gangrenous. With surgical treatment, this area sloughed off and healed three months later.

Chronic Venous Insufficiency with Ulcer. *Case 8.* P. L. T.: a 62 year-

old white male with a three-year history of pain and swelling of his left leg following an injury. For the past year, he has had an ulcer of the left leg.

Examination revealed stasis dermatitis, varicose veins, and a large ulcer in the left mid-leg.

Treatment consisted of pressure dressing. Vitamin E was given, 300 mg. orally daily. Healing occurred within 30 days.

Case 9. E. F.: a 40 year-old white male who has had varicose veins in both legs for the past 18 years. He noted an eruption over both legs for the past year.

Examination revealed infectious eczematoid dermatitis secondary to venous stasis, involving the entire right leg and in patches on the left leg.

He was treated with wet packs for one week. Vitamin E was given in doses of 300 to 600 mg. orally and 200 mg. intramuscularly daily. The infection cleared and granulation was complete within 30 days. The redness, scaling, and dryness of the skin persisted.

Case 10. C. H. S.: a 31 year-old white male who had phlebitis in the left leg after appendectomy 18 years ago. Since then he has had swelling and ulceration about the ankle. Eight years ago, he had vein injections without relief.

Examination showed brownish pigmentation over lower left leg, with small ulcer behind the internal malleolus.

Treatment consisted of local pressure dressings plus vitamin E, 400 mg. orally daily. Complete healing of the ulcer occurred in three weeks.

Case 11. J. E. N.: a 67 year-old white male with history of a chronic ulcer on his left leg for the past two months.

Examination revealed the brownish pigmentation of stasis dermatitis, with a 3 x 3 cm. ulcer in its center, on the lateral surface of the left leg.

Treatment consisted of pressure dressings and vitamin E, 300 mg. orally daily. The ulcer healed 6 weeks after admission.

Case 12. G. M.: a 29 year-old white male with history of phlebitis in the right leg four years ago, followed by ulceration. The large ulcer on the right leg was grafted 18 months ago. Both ulcers broke down one month before admission.

Examination revealed a large post-phlebitic ulcer on the posterior aspect of the right leg and a smaller ulcer on the medial aspect, near the ankle.

Treatment consisted of pressure dressings and vitamin E, 600 mg. orally daily. The small ulcer healed. The larger ulcer has shown only slight granulation 3 months after admission.

Case 13. J. P.: a 61 year-old white male with symptoms, for the past 10 years, of swollen and ulcerated legs. These ulcerations would heal and break down at intervals.

Examination showed four plus edema of both legs to the knees. The skin was red, excoriated, oozing, and markedly indurated. Two large ulcers, with a narrow ulcerated isthmus, were present on the left leg. A smaller

ulcer on the right ankle and an ulcer over the right first metatarso-phalangeal joint were also present.

With bed rest and digitalis, the edema subsided. Local treatment with wet dressings and pressure bandages was started. Vitamin E was given, 300 to 600 mg. orally and 100 mg. intramuscularly daily. The small ulcer on the right leg healed. He had two episodes of skin cellulitis, which responded to penicillin. The large ulcers are smaller but have not healed six months after admission. The patient refuses skin grafting.

Hypertensive Ischemic Ulcer. Case 14. D. F. T.: a 28 year-old white male with a history of a painful ulcer on his right mid-leg for past year. In the past month, he has developed similar painful ulcers on his left leg. There is no history of phlebitis. He has a history of hypertension for the past five years.

Examination revealed old, healed ulcers of both mid-leg areas and three ulcers now open and bleeding. There were no varicose veins. Peripheral pulses were all present.

Treatment consisted of local pressure dressings and vitamin E, 600 mg. orally daily. Complete healing occurred within 6 weeks.

Frost Bite. Case 15. E. R. K.: a 55 year-old white male who froze his feet 6 weeks before admission. While he was attempting to warm his feet in an oven, all his toes turned black. Hospitalized elsewhere, he lost all his toenails and parts of the tips of several of his toes.

Examination on admission showed varying degrees of ulceration of all his toes, with partial granulation. Peripheral pulses were all present.

Treatment consisted of local wet dressings plus vitamin E, 300 to 600 mg. orally daily. At the end of 75 days, healing was almost complete.

Comment

In the six cases of thromboangiitis obliterans, none can be considered to have shown improvement while on vitamin E. Three of the cases required amputation. One healed after the patient stopped smoking. One is healing since the patient stopped smoking and had a sympathectomy. The sixth case is showing no improvement, is still smoking, and is continuing to have thrombophlebitis, while still on vitamin E.

These results are directly attributable to the effects of smoking and the chronic course of the disease. The best therapy is ineffective while the patient still smokes.

If the patient stops smoking and is placed in bed in a warm environment and the vasospasm is controlled, then healing can be expected, provided there is adequate collateral circulation. Vitamin E has contributed nothing to our cases who have stopped smoking and has been just as ineffective in those who continue to smoke.

The single case of arteriosclerosis obliterans showed continued gangrene while on vitamin E requiring mid-thigh amputation. The stump wound was injured, became gangrenous, and healed only after 90 days of active surgical treatment, while on vitamin E.

Of the six cases of venous insufficiency with ulceration, two healed within 30 days, one required 42 days, and one healed within 21 days. The other two cases are still not healed, three and six months, respectively, after beginning treatment with vitamin E.

These results cannot be attributed to vitamin E. The healed cases required the usual length of time and the two which have failed thus far will undoubtedly require skin grafting for complete healing. Adequate treatment in such cases includes overcoming local sepsis and stimulating granulation with pressure dressing. Bed rest in the beginning is essential. Later, ambulation with elastic bandages is permitted.

One case of hypertensive ischemic ulcer and one of frost bite showed healing in 6 and 10 weeks, respectively. Such results can be achieved with accepted methods of treatment and vitamin E did not shorten the period of healing in either case.

Summary

Vitamin E, in the form of alpha-tocopherol, has failed to influence favorably the progress in cases of thromboangiitis obliterans, chronic venous insufficiency with ulceration, hypertensive ischemic ulcer, and frostbite. Each case proceeded to its expected conclusion, despite continued administration of large doses of alpha-tocopherol. Vitamin E failed to hasten healing time of leg ulcers.

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ALPHA-TOCOPHEROL IN THE MANAGEMENT OF SYDENHAM'S CHOREA*

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Introduction

The basis for this report is a study made upon 35 individuals of both sexes, ranging in age from 6 to 19 years. The study represents a 15-month period. The reasons for this investigation were several. In the first place, the accepted methods of management of chorea have not been too satisfactory; secondly, alpha-tocopherol had given promising results in the management of somewhat similar pathology in ischemic extrapyramidal systems in elderly people; thirdly, because of the high incidence of association of rheumatic heart disease with chorea, it was felt that a possible prophylactic measure might be evolved.

Symptoms

All cases studied were not severe problems. However, symptoms were sufficiently troublesome and alarming to the person involved for medical attention to be sought. The most frequent symptoms were: weakness; incoordinated, purposeless jerks of the extremities, head, and neck; poor appetite; stumbling gait; nightmares; frequent urination; "thrashing about" in sleep; and biting of nails (two cases). Twenty-two patients gave a history of recurring tonsillitis. Fourteen patients complained of knee or ankle joint aches. There were three patients who had had night sweats. Two individuals suffered from enuresis.

Objective Findings

Physical examination revealed the following data: All patients except three were of lean, angular build. All except five were underweight for their age norm; 35 per cent were taller than their height norm. All cases had a tachycardia ranging from 94 to 128. Thirty of the hearts presented a rapid 'slapping' 'tic-tock' type of mitral sound, in contradistinction to the more resonant and longer clicking normal sound. There were five blowing systolic mitral murmurs, well compensated. Fluoroscopically, heart sizes and shapes were within normal limits, but virtually all the hearts were of a vertical type. The only unusual electrocardiographic findings were the rapid rates. Thyroid, kidney, and liver study were non-contributory in all. All patients, except four, had hyperactive deep tendon-reflexes; twenty-two had hyperactive superficial reflexes; twenty-nine showed choreiform incoordinate twitchings. All patients had their tonsils present, and there were eleven who had infected tonsils when seen initially. Nine had increased sedimentation rates ranging from 18-32 mm. (Westergren method). There were two anemias of a microcytic hypochromic type.

* "Gelucaps Vascuals" (VCA) an enteric, coated, emulsified, natural high potency alpha-tocopherol was kindly furnished by the Vitamin Corp. of America, Newark, for this study.

† Much bibliographic assistance in basic tocopherol investigation was generously furnished by Miss Sophie R. Gordon, M.A., of the Gordon Wheat Germ Company, New York City, mgr. of "Ecentrate."

Method of Investigation

Every patient had been previously treated by the usual method. This consisted of sedation with one of the barbiturates, bed rest, and multi-vitamin therapy. This regimen had not proved very helpful. For treatment purposes in our study, patients were divided into two groups. A control group consisting of every other patient was placed upon a placebo. The second group was placed upon 90-225 mg. daily of natural alpha-tocopherol, divided into three equal doses. No other medication was used, except that the anemic patients were given iron sulphate to correct the anemia before institution of tocopherol therapy (iron salts destroy the biological effect of alpha-tocopherol).

Results

In the controls (17 patients), there was symptomatic improvement in two patients. In the treated group, there was amelioration in the symptoms of all: all slept less fitfully; choreiform motions were abolished in 13 out of 18 treated cases; appetite was improved in all tocopherol-treated anorexics; joint symptoms disappeared. After four weeks, the treated group were virtually all free of their initial complaint, with the exception of one case of persistent enuresis. There was not much change in the status of the control group. Whereupon, the latter group was placed on tocopherol therapy, and, within 2-5 weeks, all patients were virtually asymptomatic. The one persistent case of enuresis was referred and is under treatment by a child psychiatrist. Objectively, the under weight group gained from 4 to 10 pulse rate dropped to 80-110 (in contrast to initial 94-128); heart sounds were less shallow in quality and more vigorous in all hearts; fluoroscopically, there was no change; electrocardiographically, there was no change; deep tendon reflexes had reverted to normality in 29 patients. All joint tenderness had disappeared. All patients have had tonsillectomies, and, at the date of this writing, with the exception of one case (enuresis case), all are virtually symptom free.

Case Reports

There follow two case histories characteristic of this series. *Case 1.* G. F., age 11, was first seen by us because of anorexia, spasmodic jerks of the head, purposeless incoordinate twitching of the hands, and an awkward gait of several months duration. There were also night sweats, nightmares, and 'tossing and thrashing about' in bed at night of similar duration. Past history revealed the occurrence of measles, mumps, and whooping cough at the ages of 5, 6, 7, years, respectively. Past history was otherwise non-contributory. Physical examination revealed a tall (63 inches) emaciated (88 pounds) lad, who was very fidgety and in no acute distress. His build was lineal and angular; his head and face were dolichocephalic and leptoproscopic, respectively. Other significant observations were: enlarged, engorged, tonsils; pulse rate, 116 beats per minute; heart sounds, rapid, with slapping, diminished intensity at all valvular areas. Fluoroscopic and electrocardiographic studies were non-contributory except for a tachycardia. Thyroid, renal, hepatic, and gastro-intestinal studies were nega-

tive. Knee, ankle, biceps, and triceps jerks were moderately hyperactive (Grade ii). There was a coarse tremor elicited in the finger tips of both hands, and a coarse incoordinate jerk in both arms and head. Balance studies were negative, as was his Babinski.

The patient was started on 225 mg. of alpha-tocopherol daily, in three equally divided doses. After three weeks, the patient was re-examined and the following results were noted: Incoordination and spasmodic twitching had disappeared. He no longer had nightmares or night sweats and slept less fitfully. The intensity of heart sounds improved and his pulse rate dropped to 86 beats per minute. A tonsillectomy was then performed, and the patient is now symptom free.

Case 2. P. N., age 8, was seen initially because of chief complaints of nightmares, emaciation, spasmodic twitching of the head, weakness, and anorexia of some three months duration. Past history revealed that this little girl had had measles and mumps at the age of 6. There had been an appendectomy at the age of four. Otherwise, past history was non-contributory. Physical examination revealed a tall (51 inches) puny youngster (weight 59 pounds) of an angular build. Head and facial indices fell within the dolichocephalic and leptoprosopic ranges. Tonsils were present but were not remarkable. Heart sounds were of diminished intensity and of rapid, slapping character. Pulse rate was 104. Electrocardiogram and fluoroscopic study were negative except for a tachycardia. Thyroid, hepatic, renal, and gastrointestinal studies were negative. Tendon reflexes, as well as superficial skin reflexes, were moderately hyperactive. There was an involuntary choreiform jerk noted in the head. Balance function tests were normal. Urine was negative; blood picture was essentially normal, except for an elevated sedimentation rate—23 mm. (Westergren method).

After three weeks of control therapy with placebos, the patient was re-examined and no favorable changes were noted. She was then placed upon 150 mg. of alpha-tocopherol daily, divided into two equal doses. This case was evaluated again after a month, and the following findings were elicited: initial symptoms had abated; pulse rate had dropped to 86; heart sounds were of better quality and more vigorous; and reflexes had reverted to virtual normalcy. The youngster had gained 7 pounds, and her sedimentation rate had dropped to normal. A tonsillectomy was then performed, and, at the present moment, the youngster has no complaints.

Discussion

Experiences with 35 patients suffering from Sydenham's chorea have just been presented. From a consideration of results obtained with alpha-tocopherol therapy it appears that this vitamin is a useful agent in revascularizing and restoring to function ischemic extrapyramidal systems in individuals who have a tendency toward the rheumatic syndrome. It is felt that alpha-tocopherol might prove very useful: (1) in the treatment of the very troublesome symptom complex of chorea; and (2) in the prevention of rheumatic endocarditis in children.

MASSIVE DOSAGE OF ALPHA-TOCOPHEROL IN ALLEVIATION OF MULTIPLE SCLEROSIS*

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Introduction

Seven patients, ages 32-47, who have been afflicted 31-240 months, have been treated, as private patients, for 3-15 months. Our basis for an attempt at treatment with vitamin E lay, first, in three papers by Wechsler,¹ Bicknell,² and Davison³ in which improvement was noted, both clinically and histologically, in amyotrophic lateral sclerosis. Histologically, the demyelination and gliosis⁴ of multiple sclerosis is similar to the aforementioned syndrome. Secondly, in our geriatric neuropathologies, with similar histopathologic pictures but less extensive involvement, there was some amelioration of symptoms. It was felt that, possibly, some reversal, with attendant increased function, might be effected in nerve tissue which had not yet become fully gliotic. The report is very incomplete, and no definite conclusions may be drawn. It is "thrown out" to the profession to stimulate further study, especially in view of the natural history and apathetic therapy of this disease.

Symptoms

Symptomatology has been protean, but the chief difficulties have been ataxia, spasticity, muscle weakness, hyper- and paresthesias, speech and visual difficulties (1 case), and 70 per cent deafness.

Objective Findings

Physical examination revealed the usual picture of intention tremors, asymmetrically hyperactive deep tendon reflexes, loss of abdominal reflexes, positive Babinskis, muscular hypotonia or atrophy, and a wide range of sensory abnormalities. The physical examinations were otherwise non-contributory. Incoordination was present in all patients, and vibratory sense was absent in the lower extremities of two patients.

Method of Treatment

Because of the anti-ischemia properties of alpha-tocopherol, it was thought some improvement might be effected by its use. We used and recommend the following method of management:

(1) The patient was given 300 mg. of "Vita E Injectable" (Vitamin Corp. of America, Newark) intramuscularly daily, in three equal 100 mg. doses (first day, one-half the amount was used). This was continued for 4-7 days, dependent upon the size of the patient and the reaction. No iron

* "Gelucaps Vasculals" (VCA), an enteric, coated, emulsified, natural high potency alpha-tocopherol, was kindly furnished by the Vitamin Corp. of America, Newark, N. J., for this study (100 mg. capsules).

† Much bibliographic assistance in basic tocopherol investigation was generously furnished by Miss Sophie R. Gordon, M.A., of the Gordon Wheat Germ Company, New York City, Mfr. of Ecentrate.

may be used with E. Hypertensive and hyperthyroid patients should not receive it because of slightly thyrotropic action and mild initial blood pressure elevation in some cases.

(2) Simultaneously, for relaxant effect, the patient received *one* of the following antispastics: prostigmine, tolserol, tubocurarine in oil (Abbott), vinobel, or an antihistaminic. Adequate dosage and necessary precautions should be taken, dependent upon the preparation selected.

(3) The patient received high potency vitamin B complex. We used "Provite B-IVC" or "Combex" with C (2) (P.D.) t.i.d., depending on the patient's tolerance.

(4) The patient received crude liver extract (Armour), 2-4 Units per week I.M.

(5) The patient received treatment for any other unrelated pathology by appropriate therapy; for instance, diet and methionine, as a lipotrope, were employed in a fatty infiltrated liver.

(6) After the first week, the patient was placed upon 400-600 mg. of alpha-tocopherol daily in divided doses which were multiples of the 100 mg. capsule. The patient was maintained on other supportive therapy (B-Complex, relaxant, and liver extract).

(7) After two weeks, the supportive therapy was reduced to a maintenance level; the alpha-tocopherol was maintained at a high level; and good corrective muscle and nerve coordination re-education was begun. The "twilight zone" (areas where the nerve tissue is neither completely gliotic nor yet functional) degenerating nerve tissue had been "primed" and re-education of the type used in the Veteran Hospital paraplegic and hemiplegic cases was instituted.

(8) The subsequent courses of therapy depended upon the response of the individual patient. In chronic cases, at least 9 months should elapse before any definite trial can be considered fruitless.

(9) After 3 months, the patient was maintained on 300-600 mg. of alpha-tocopherol. Here again, dosage was an individual problem.

(10) After relaxation was obtained, so that the corrective therapist could maneuver the muscles, the relaxant was decreased or discontinued.

Results

The results were quite revealing. Two cases reverted to virtual normalcy (a few residual paresthesias were left) after 5 and 9 days, respectively, of therapy. These were acute types, which were seen a few days after onset. Each had had several previous bouts which had lasted for 2-2½ months. The disease had been first noted 2 years before in one and 2½ years in the other. The remaining 5 cases were chronics (5-10 years). After four weeks of tocopherols, there was less muscle weakness, less atrophy, and decreased paresthesias and hyperesthesias, deafness disappeared, and better coordination in locomotion was present. After 2-14 months of corrective therapy, along with maintenance tocopherols, ataxia, spasticity, and muscular strength have been moderately improved in 3 patients. Two severe cases have been unaffected as yet.

Discussion

The above program is entirely empirical and based essentially upon the use of alpha-tocopherol for over three years upon the ravages of arteriosclerosis, as seen in a geriatrics clinic. There, after objective observation of the results of supportive agents in ameliorating arteriosclerotic diseases, alpha-tocopherol was added and appeared to improve patient status quite impressively and quite often. In multiple sclerosis, the pharmacological effect appears to work likewise when the combination is used.

Finally, alpha-tocopherol appears to be a useful tool, *but not a panacea*, which needs further study before its rôle can be accurately appraised. From a study of the treatment just described, we feel that massive doses of alpha-tocopherol, when used in conjunction with good corrective therapy, offer a worthwhile approach to the management of multiple sclerosis.

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TOCOPHEROL THERAPY OF URETHRAL STRICTURES—A PRELIMINARY REPORT*

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Favorable therapeutic results have been obtained from the administration of alpha-tocopherol for a number of clinical diseases.^{1, 2, 3} Prominent in the field of urology is the recent use of tocopherols for Peyronie's Disease. Scott and Scardino have demonstrated that certain patients who have this disease respond favorably in that both the distressing symptoms and the primary fibrositis can be alleviated.⁴

The methods and agents which are available for the treatment of urethral strictures are, in essence, those which have been used for more than fifty years. Dilatation with flexible or rigid instruments, internal urethrotomy, open surgical excision—these and other similar procedures are the ones most commonly relied upon for the maintenance of an adequate channel, once a stricture of the urethra has developed. The inadequacy of such measures is too well appreciated to require comment. Effective medicinal measures, except for those directed toward control of urinary tract infection, have not previously been developed.

It has seemed reasonable to assume that tocopherols might resolve the scarring of secondary fibrositis. Urethral strictures represent such a fibrositis. We have had two years' experience with treating urethral strictures with mixed tocopherols and believe that the results warrant a brief review of our observations.

Experimental

The clinical material for this study consists of both public ward and private outpatients under the care of the authors and other members of the staff of the Brady Urological Institute. A group of twenty-two cases, in which appraisal is feasible and follow-up periods are adequate, form the basis for the present study. Urethral strictures are of varied etiology. Postoperative, post-traumatic, postgonococcal, and congenital strictures have been treated in our series. In every instance, patients have been carefully evaluated prior to the institution of therapy. The urethra has been calibrated, symptoms analyzed, urinary stream observed, and past urological histories reviewed.

Following the pre-treatment evaluations, vitamin E in the form of mixed tocopherols has been administered orally. In the earlier cases, a daily dosage of 200–300 mg. of mixed tocopherols (representing 100–150 mg. of alpha-tocopherol) was employed. Dosage as high as 1200 mg. daily has been used in several cases.

Follow-up periods of almost two years have been obtained for some patients, but significant cases treated or followed for a shorter length of

* Both "Eprolin" (Eli Lilly) and "Tocopherex" (Squibb) were generously contributed by the manufacturers and were the only sources of tocopherols used in this study.

time have not been excluded from this report. No cases have been eliminated from consideration for purely arbitrary reasons.

During and after the course of tocopherol administration, patients have been examined at frequent intervals. In most instances, instrumentation and calibration of the urethra has been performed at these visits. In some cases, no instrumentation or dilatation has been utilized in conjunction with the tocopherol therapy.

Results

Good results were obtained in 15 of the 22 cases treated with tocopherols, and fair results in 4 cases. Three patients made no detectable response to therapy. The etiology of strictures does not seem to alter the therapeutic response (TABLE 1), nor does the duration of the stricture appear to be of

TABLE 1

<i>Etiology of strictures</i>	<i>No. of cases</i>	<i>Response to therapy</i>		
		<i>Good</i>	<i>Fair</i>	<i>No response</i>
Postoperative	5	4	0	1
Post-infectious	12	9	2	1
Post-traumatic	2	1	1	0
Congenital	1	0	1	0
Other	2	1	0	1
Totals	22	15	4	3

importance. Strictures of over 30 years standing have been treated successfully with tocopherols.

The desirable length of therapy and the optimal dosage of vitamin E cannot be established by perusal of these cases. It is, however, of interest that several strictures responded either to prolonged administration of tocopherols or to an enormous increase in the daily dosage, cases which initially showed little or no improvement.

Many of the patients given a trial on tocopherol had previously had the benefit of every other conceivable form of treatment. Several had previously had suprapubic cystostomy and retrograde dilatation. Nine patients had suffered bouts of acute urinary retention. Twelve strictures were completely impassable at sometime during the period of observation. Impassable stricture has recurred in only one instance following tocopherol therapy. In five cases, instrumentation was arbitrarily omitted during the time tocopherol was administered. These are perhaps the most significant ones. It seems permissible to ascribe the pronounced improvement in such patients to the medication.

Summary

(1) The rationale for treating urethral strictures of varied etiology with orally administered vitamin E is briefly considered.

(2) A series of 22 urethral stricture patients who have been given 200 to 1200 mg. of mixed tocopherols daily (i.e., 100-600 mg. of alpha-tocopherol) has been described.

(3) Favorable response to tocopherol therapy, both with and without concomitant dilatation, has been observed in more than one-half of the patients treated.

(4) Continued use of vitamin E for urethral strictures seems justifiable.

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